

COMPARATIVE STUDIES ON THE QUATERNARY
STRUCTURE OF FERRITIN

by

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ABBREVIATIONS

| | |
|----------------|-------------------------------------|
| PAGE | Polyacrylamide gel electrophoresis |
| bis | N, N' methylenebisacrylamide |
| Temed | N, N, N', N', tetramethylenediamine |
| SDS | Sodium dodecyl sulphate |
| EDTA | Ethylenediaminetetra-acetic acid |
| IEF | Isoelectric focusing |
| PI | Isoelectric point |
| GEF | Gel electrofocusing |
| DTT | Dithiothreitol |
| cm-cellulose | Carboxymethyl cellulose |
| DEAE-cellulose | Diethylaminoethyl-cellulose |

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CHAPTER I

GENERAL INTRODUCTION

1.1 The Discovery and Early Work on Ferritin

The initial discovery of the iron-storage protein ferritin is attributed to the German pharmacologist Naunyn Schmiedeberg (1894), who described a protein which he called ferratin. Isolated from pig liver, this molecule was shown to contain 6% iron and a variable amount of phosphate. Pure ferritin, a water soluble molecule containing 20% iron by weight was first isolated from horse spleen by Laufberger (1937).

Throughout the 1940's, the method of isolation and properties of ferritin were studied by Hahn, Granick and Michaelis. Kuhn *et al.* (1940) repeated Laufberger's experiments and considered this iron containing protein to be a well-defined chemical entity containing 50% protein, 12% nucleic acid and 35% iron hydroxide. Granick (1942) and Granick and Michaelis (1945) made a thorough study of ferritin, and were not able to confirm the presence of nucleic acid as reported by Kuhn *et al.* (1940).

The function of ferritin as an iron-storage protein was demonstrated by Hahn *et al.* (1943), who showed that radioactive iron administered as ferric ammonium citrate by vein to the dog was converted into ferritin iron in the liver. They also showed that iron derived from haemoglobin of the red blood cells, following destruction

of the cells by acetylphenylhydrazine, was in part converted to ferritin iron in the liver and the spleen.

Until 1948, the only reported function of ferritin was that of iron storage. Mazur and Shorr (1948) then proposed a role for ferritin in the regulation of the peripheral circulation through a vasodepressor activity. Antidiuretic properties were also attributed to ferritin (Baez *et al.*, 1950). It was suggested (Baez *et al.*, 1952) that ferritin antidiuretic activity resulted from ferritin stimulation of the neurohypophysis to secrete anti-diuretic hormone, and that the vasodepressor activity resulted from inhibition of constrictor response to topical epinephrine. In recent years however, this subject has attracted no attention, and the fact that ferritin in serum may exceed 1 mg/l without causing any of the symptoms observed in Mazur's laboratory, casts doubt on the physiological significance of these observations.

Much of the other work carried out in the 1950's was concerned with the structure of ferritin, and ferritin metabolism.

1.2 Ferritin Structure

Rothen (1944), while investigating the properties of

ferritin in the ultracentrifuge, found that ferritin consisted of a complex; about 25% free apoferritin, and ferritin with a broad distribution of iron content. Apoferritin was found to be a homogeneous protein with a sedimentation constant of 17.6 S. Ferritin and apoferritin had the same electrophoretic mobilities and isoelectric points, indicating that the iron was probably sequestered inside the molecule (Rothen, 1944; Mazur and Shorr, 1950). This was confirmed by electron microscopy (Farrant, 1954), and later by X-ray diffraction (Harrison, 1959).

The protein has a diameter in the order of 120 Å, while the iron micelle has a diameter of 70-75 Å (Farrant, 1954; Harrison, 1963; Bielig *et al.*, 1966; Kleinwachter, 1964; Fishbach and Andéregg, 1965). Fishbach and Andéregg (1965) isolated "full" ferritin by density centrifugation and calculated that the micelle contained 4,300 iron atoms per molecule.

The Iron Core

The iron oxide core has sufficient density to be seen in electron micrographs without prior staining. Farrant (1954) suggested that the iron was distributed in four electron-dense regions. Later, Muir (1960), Bessis and Breton-Gorius (1960), and von Bruggen *et al.*, (1960) reported that the various core patterns seen in electron

micrographs could best be accounted for by assuming that they represented projections in varied orientation of molecules containing six electron-dense regions arranged either at the vertices of an octahedron (Muir, 1960; Bessis and Breton-Gorius, 1960) or at the vertices of a trilateral prism (von Bruggen *et al.*, 1960).

However, electron microscopy of a sufficiently high resolution shows that the iron core does not appear to have a uniform structure. Haggis (1965) obtained electron micrographs with resolution better than 4 Å which suggested that the iron oxide was present in ferritin molecules in the form of small crystallites of varying numbers in different molecules, more or less randomly distributed within the protein shell. Massover and Cowley (1975) used dark field microscopy at high voltage to obtain high resolution images of ferritin cores, which showed that some cores are a single large crystallite, while others are composed of one or several small crystallites which are not geometrically related to each other.

The Protein Shell

The molecular weight of horse spleen apoferritin was first estimated to be 465,000 from an analysis of its rates of sedimentation and diffusion (Rothen, 1944). From X-ray diffraction studies (Harrison, 1959), and density

measurements (Harrison, 1963), a molecular weight of 480,000 was obtained. However these determinations were based on the measured partial specific volume \bar{v} of 0.744. Using \bar{v} calculated from the amino acid composition, 0.731-0.733 (Harrison, 1969; Crichton *et al.*, 1973) the above weights become 440,000 and 450,000 approximately. Low angle X-ray scattering (Bielig *et al.*, 1966) gave a value of 462,000, while light scattering (Richter and Walker, 1967) gave molecular weights between 430,000 and 479,000. The approach to sedimentation equilibrium, the meniscus depletion method of Yphantis (Bjork and Fish, 1971) gave 465,000, and true sedimentation equilibrium (Crichton *et al.*, 1973) gave 443,000. As can be seen, the molecular weights for horse spleen apoferritin obtained by the different methods described above appear to agree extremely well, giving values between 430,000 and 470,000. The molecular weight of apoferritins from other tissue sources may differ. This is shown in a study of rat tissue ferritins (Vulmiri *et al.*, 1975), where the following molecular weights were obtained: rat liver 493,000; rat hepatoma 443,000; rat kidney 421,000 and rat heart 531,000.

That apoferritin consists of subunits and not one long polypeptide chain was first demonstrated by Hofmann and Harrison (1963). A discussion of the subunit structure of ferritin will follow in Section 1.6, but to enable a

clear understanding of the ensuing description of ferritin metabolism, it is necessary to appreciate that ferritin consists of a protein shell composed of a number of polypeptide chains surrounding a matrix of iron oxide and phosphate, much in the same way as viral protein encapsulates viral nucleic acid.

1.3 Ferritin Metabolism

Ferritin Biosynthesis

Speed of uptake of iron and its conversion into ferritin iron were studied by Granick and Hahn (1944). These authors observed that one hour after injecting rats with radioactive ferric ammonium citrate (containing about 0.45 mg iron), over 40% of the injected iron was present in the liver, while after two hours 60% was present in the liver. However, it was not fully appreciated until 1955 that iron actually regulated ferritin biosynthesis through *de novo* stimulation of apoferritin synthesis (Fineberg and Greenberg, 1955a). Intraperitoneal injection into guinea pigs of 1.5 mg of iron per kg body weight as ferric ammonium citrate produced a several fold increase in incorporation of labelled carbon from leucine or glycine into liver ferritin. The lack of any comparable effect of iron on uptake of ^{14}C into mixed liver protein ruled out any interfering effect of iron on pre-

cursor pools and permitted the conclusion that the increased uptake of ^{14}C indicated accelerated synthesis of ferritin (Fineberg and Greenberg, 1955a). The increase in ferritin was not due to stabilisation of the protein against breakdown by iron, since the activity of ^{14}C was greater in apoferritin than in ferritin (Fineberg and Greenberg, 1955b).

Drysdale and Munro (1966) showed that the *in vivo* incorporation of labelled amino acids into rat liver ferritin increased to a maximum at about 6 hours after intraperitoneal injection of ferric ammonium citrate, and returned to a basal level after twelve hours. The effect was not inhibited by prior administration of actinomycin D, indicating that the iron induction of apoferritin biosynthesis occurred at a post-transcriptional stage.

In vitro, liver microsomes from iron treated rats were shown to incorporate labelled amino acids more extensively into ferritin than those from control rats, and this effect was not diminished by previous treatment of the rats with 70 μg actinomycin D/100 g body weight (Saddi and von der Decken, 1965).

Iron stimulation of ferritin biosynthesis was also shown in rat liver slices (Yu and Fineberg, 1965; Yoshino *et al.*, 1968). However in these experiments, the prior administration to the rats of 1800 μg actinomycin D/100 g

body weight (Yu and Fineberg, 1965), and 400 µg actinomycin D/100 g body weight (Yoshino *et al.*, 1968) prevented increased incorporation of labelled amino acids into ferritin due to iron. In neither of these investigations was the effect of the large dose of inhibitor on general protein synthesis examined. Massive doses of actinomycin D do in fact reduce uptake of ^{14}C into ferritin in liver slices from iron-treated rats, but incorporation into mixed liver proteins is depressed to a similar extent, indicating a general toxic action that could explain the apparent suppression of ferritin induction by the inhibitor (Drysdale and Munro, 1966). Cycloheximide completely abolished the stimulating effect of iron on ferritin biosynthesis (Chu and Fineberg, 1969; Millar *et al.*, 1970), thus indicating that protein synthesis is required for the iron effect.

Ferritin is synthesised on free polyribosomes rather than membrane bound polyribosomes (Redman, 1969; Hicks *et al.*, 1969; Puro and Richter, 1971), although Puro and Richter (1971) did detect synthesis of some ferritin on membrane bound polyribosomes. More recently, Lee and Richter (1977) reported that ferritin protein was synthesised on both free and membrane bound polyribosomes, and that free polyribosomes synthesise substantially more ferritin than membrane bound polyribosomes.

A two-fold increase in the polysomal ferritin mRNA content has been shown to be involved in iron induction of ferritin synthesis (Zahringer *et al.*, 1975). A model has been proposed (Zahringer *et al.*, 1976) in which translation of ferritin mRNA is prevented by adhering ferritin subunits. Iron administration removes this inhibition of the translation by promoting aggregation of these subunits into ferritin, thus allowing translation to occur.

Iron Uptake and Release

An early model to describe how iron entered ferritin was proposed by Pape *et al.* (1968). Ferritin formation according to this model takes place by the polymerisation of ferric chelates to form micelles independently of the protein moiety. The complete apoferritin is in equilibrium with its subunits, and the iron micelles are trapped as the apoferritin subunits assemble to form the shell. The protein subunits are then stabilised by their arrangement around the iron. The main criticism of this model is that when ferritin synthesis is induced by iron, the first molecules to be formed are poor in iron, while if ^{59}Fe is included in the inducing dose, the label gradually passes to iron-rich fractions over a period of 72 hours (Fineberg and Greenberg, 1955b; Drysdale and Munro, 1966; Macara *et al.*, 1972). These latter experiments

indicate that ferritin is formed by progressive iron accumulation.

That oxidation of ferrous iron is involved in iron uptake by ferritin has been demonstrated (Bielig and Bayer, 1955; Loewus and Fineberg, 1957). A "synthetic ferritin" (the crystallites were not as large as in native ferritin) could be obtained from the incubation of horse spleen apoferritin in bicarbonate buffer pH 7.4-7.6 with ferrous ammonium sulphate, air oxidation at 4-6°C, and subsequent crystallisation from CdSO_4 (Bielig and Bayer, 1955). These results were confirmed with rat and horse apoferritins (Loewus and Fineberg, 1957), while it was also possible to show that ferric ammonium citrate could serve as a source of iron for ferritin in the presence of a rat liver extract or of ascorbate and molecular oxygen. However the quantity of iron incorporated from ferric iron amounted to only 15% of the original iron content of the ferritin from which the apoferritin had been prepared, whereas uptake from ferrous iron was found to be 80% of the original iron content of the ferritin. Uptake of ferrous iron by apoferritin was also found in the presence of suitable oxidising agents or molecular oxygen (Harrison *et al.*, 1967), and apoferritin was reported to influence the rate of oxidation of ferrous iron as well as the structure of the micelle produced.

Niederer (1970) showed by following the loss of Fe^{2+} , that apoferritin catalysed the oxidation of Fe^{2+} to Fe^{3+} in marked contrast to a number of other proteins. A slight decrease in oxidation occurred after treatment of apoferritin with formaldehyde, β propiolactone or bromoacetate, and a strong inhibition of the catalysis by Zn^{2+} . He postulated that ferritin formation takes place by penetration of the apoferritin shell by Fe^{2+} ions, which are oxidised at the inner surface of the protein by a catalytic active site: the Fe^{3+} formed an intramolecular precipitate which very soon became too large to escape from the apoferritin shell. He further postulated that histidine residues in the protein were involved in the catalytic site.

Macara *et al.* (1972) extended the observations of Niederer (1970) and found that the progress curves for iron uptake by apoferritin were sigmoidal, whereas those for ferritins of low iron content were hyperbolic. They proposed a "crystal growth" model in which the surface area of the crystallite forming inside the protein increases until the molecule is half full, and then declines. The surface area of the crystallite controls the rate at which new material is deposited. Macara *et al.* (1972) also favoured a model in which the oxidation site is also the site at which the core starts to grow; in this model oxidation and nucleation can be regarded as

a single step.

Crichton and Bryce (1972) and Bryce and Crichton, (1973) found a hyperbolic progress curve for the oxidation of Fe^{2+} by apoferritin, and the increase in initial velocity was linear with increasing apoferritin concentration. With respect to Fe^{2+} the reaction followed Michaelis-Menter kinetics. In contrast to Macara *et al.* (1972), Crichton (Bryce and Crichton, 1973) proposed that the oxidation and nucleation was not a one step reaction but rather a two step reaction. Oxidation would occur at sites between the subunits, where two ferrous ions would bind, and the subsequent reaction would involve formation of a superoxide before Fe^{3+} was produced and released into the inside of the protein shell, where nucleation would occur at a separate site.

The question as to whether oxidation of Fe^{2+} to Fe^{3+} occurs in the interior of the molecule or between the subunit channels has yet to be resolved.

Mobilisation of iron from ferritin can occur by way of low molecular weight reductants, low molecular weight chelators and by an enzymic mechanism. Biological reductants such as ascorbate, cysteine and glutathione can all reduce ferritin, but at rates which seem too slow to be of physiological significance (Bielig and Bayer, 1955; Sirivech *et al.*, 1974; Dognin and Crichton, 1975).

Cysteine was found to be the most effective in iron mobilisation but concentrations in excess of 0.05 M were required and this high concentration is itself unphysiological (Dognin and Crichton 1975).

Of the low molecular weight chelators, both nitrilotriacetate and EDTA have been shown to mobilise iron from ferritin without any requirement for reduction. However, although 40% of ferritin iron could be mobilised in 120 hours by nitrilotriacetate, the only physiological chelating agent used, sodium citrate, released 1% of the ferritin iron in this time (Pape *et al.*, 1968). Glucose, fructose, glycine, succinate, citrate and AMP released very little iron from ferritin, the best being fructose (Dognin and Crichton, 1975).

That iron mobilisation from ferritin might be mediated by an enzyme was first proposed by Green and Mazur (1957) Xanthine oxidase was reported to reduce ferritin under anaerobic conditions in the presence of hypoxanthine or xanthine (Green *et al.*, 1957; Mazur *et al.*, 1958; Tanaka, 1956). According to Mazur's theory release of iron from hepatic ferritin occurs during hypoxia, when red blood cell synthesis is stimulated. The decreased oxygen supply to the liver cell causes an accelerated breakdown of nucleotides including ATP, yielding increased cellular levels of hypoxanthine and xanthine, and a consequent

increase in oxidation of these substrates by xanthine oxidase. This early theory linking ferritin iron reduction to xanthine oxidase activity, has now been rejected. No mobilisation of ferritin iron was observed with xanthine oxidase *in vitro* (Crichton, 1973), while both Kozma *et al.*, (1967) and Grace *et al.*, (1970) have shown that allopurinol inhibition of xanthine oxidase does not affect iron mobilisation.

Preliminary reports of a ferritin reductase (ferriductase) activity in mammalian liver which was dependent on FMN and NADH (Osaki *et al.*, 1971), were followed by the observation that flavins in the reduced form were capable, under anaerobic conditions, of the rapid and quantitative reduction of ferritin iron (Sirivech *et al.*, 1974). Crichton *et al.*, (1975 (b)) studied the ferritin iron reducing activity in rat liver extracts and observed that FMN in the presence of NADH constituted a system which was capable of complete and rapid reduction of ferritin iron *in vitro*. They concluded that the source of electrons for ferritin reduction was ultimately NADH and the FMN functioned as a coenzyme, which might be associated with ferritin. Iron mobilisation could be regarded as a two step reaction; generation of FMNH₂ from NADH followed by reduction of ferritin iron. The ferritin reductase activity found in subcellular fractions of liver extracts (Crichton *et al.*, 1975a) more probably reflects the

distribution of an NADH-NADPH dependent FMN reductase rather than ferriductase.

While many major advances, as described above, have been made in the understanding of the processes involved in the regulation of ferritin biosynthesis, and in the mechanism of iron uptake and release from ferritin, a great deal of research over the same period of time, has also gone into the phenomenon of ferritin heterogeneity, and into the clinical implications of serum ferritin.

1.4 Ferritin Heterogeneity

Although early work had indicated that both ferritin and apoferritin migrated homogeneously in an electric field (Rothen, 1944; Mazur and Shorr, 1950), the introduction of refined electrophoretic techniques employing starch gel and polyacrylamide, made it possible to show that both ferritin and apoferritin isolated from a number of mammalian species gave multiple bands (Saddi, 1962; Richter 1963a, 1963b, 1964; Kopp *et al.*, 1963; Theoren *et al.*, 1963; Suran and Tarver, 1965). By ultracentrifugation, three components were found in horse spleen apoferritin, which corresponded to the three major fractions identified by gel electrophoresis (Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp *et al.*, 1966).

It appeared that this heterogeneity resulted from the presence of dimers and higher oligomers of a protein monomer. The hypothesis, that in the oligomers, monomer units were firmly and stably linked by covalent bonds (Harrison and Gregory, 1965) was abandoned because a variety of reagents that attack disulphide, peptide or ester linkages were found not to affect the distribution of monomers and oligomers of horse spleen ferritin or apoferritin in aqueous solutions (Williams and Harrison, 1968). Concentrations of dioxane up to 50% also failed to dissociate oligomers of horse spleen ferritin into monomers, so that the role of hydrophobic interactions was uncertain (Williams and Harrison, 1968). Hydrogen bonding seemed unlikely because of the failure of 7 mol/l urea and 6 mol/l guanidine hydrochloride or high concentrations of various salts to cause dissociation of the oligomers (Williams and Harrison, 1968).

Richter and Walker (1967) calculated the association constant of horse spleen apoferritin at 25°C from light scattering data. The values found indicated the presence of intermolecular forces weaker than covalent bonds but stronger than hydrogen bonds or van der Waal's forces. The values were consistent with reversible association and dissociation. Jaenicke and Bartmann (1972) noted concentration-dependent dissociation of oligomers by analytical ultracentrifugation, but Bjork (1973)

did not detect this. However Lee and Richter (1976) reported results of studies on the aggregation of ferritin and apoferritin molecules in solutions *in vitro*, which indicated that monomer association, and oligomer dissociation was concentration dependent.

A second type of electrophoretic heterogeneity is a structural heterogeneity giving rise to the term isoferritin. The first demonstration of isoferritins was by Richter (1965), who showed electrophoretic differences in ferritins isolated from human liver and Hela cells. Similar electrophoretic differences were subsequently found in ferritins from several normal human tissues (Alfrey *et al.*, 1967). They identified three different ferritins; one from spleen, another from liver and a third from reticulocytes. Two types of ferritin were also found in bone marrow; a fast migrating marrow ferritin (similar to that in reticulocytes, and a slow marrow ferritin (similar to that in spleen). It was proposed that the fast marrow ferritin was that in erythroblasts and that the slow marrow ferritin was the ferritin in the marrow reticulum cells (Alfrey *et al.*, 1967). Heterogeneity between different tissue ferritins has also been observed in other laboratories (Crichton *et al.*, 1973; Gabuzda and Pearson, 1969; Linder and Munro, 1973; Powell *et al.*, 1975).

A third type of heterogeneity can be demonstrated by the use of isoelectric focusing, which enables resolution of ferritins with a difference in isoelectric point of 0.05 pH units. This "microheterogeneity" of ferritins from a single organ (Drysdale, 1970), has been demonstrated by focusing in sucrose gradients, ferritins from normal rat liver (Makino and Kono, 1969; Urushizaki *et al.*, 1973) rabbit liver ferritin (von Kreel *et al.*, 1972), horse spleen ferritin (Drysdale, 1970; Urushizaki *et al.*, 1971) and human liver and spleen ferritin (Drysdale, 1970; Worwood *et al.*, 1975). Studied by focusing in polyacrylamide gels, ferritins from human liver, spleen, heart, pancreas and kidney were found to contain at least five isoferritins in most tissues, with two common to all tissues (Powell *et al.*, 1974, 1975; Drysdale *et al.*, 1975). Liver and spleen ferritins which had similar mobilities on polyacrylamide gel electrophoresis had similar focusing patterns. On the other hand, heart and kidney ferritins which were readily distinguishable from liver and spleen ferritins by polyacrylamide gel electrophoresis had more acidic isoelectric focusing profiles (Drysdale *et al.*, 1975).

Many of these isoferritins have also been resolved by ion exchange chromatography. Theoren *et al.*, (1963) and Suran and Tarver (1965) obtained three chromatographic fractions from horse spleen ferritin by DEAE-cellulose

chromatography, while Yu and Fineberg (1965) obtained three chromatographic fractions for rat liver ferritin. Horse spleen apoferritin was resolved into five fractions on DEAE-sephadex A-50, and each fraction when analysed by isoelectric focusing corresponded to different portions of the isoferritin profile (Drysedale 1974).

The iron content of isoferritins differs, although there is no clearly discernible pattern to the distribution of iron. Overall, the ends of the isoferritin spectrum tend to have the lowest iron content (Ishitani *et al.*, 1975b).

In disorders involving iron overload, changes have been observed in the isoferritin profiles. In haemochromatotic liver, most isoferritins have the same iron content (Powell *et al.*, 1974), while in both haemochromatosis and siderosis the acidic bands disappear and all the organs give liver-like isoferritin profiles. After treatment, the heart and kidney patterns revert to the normal heart and kidney type (Powell *et al.*, 1975 b). These observations were not supported by Bomford *et al.* (1977) who found that ferritins in normal heart are not confined to the acidic part of the spectrum, and that there was no consistent pattern of isoferritin distribution in iron overload.

Studies in both animal and human tissue indicate that

many cancers are characterised by an isoferritin profile of a more acidic nature than those in adult liver (Alpert *et al.*, 1973; Drysdale and Singer, 1974; Linder *et al.*, 1970; Marcus and Zinberg, 1974; Makino and Kono, 1969; Richter, 1965 and Urushizaki *et al.*, 1973). Similar isoferritins have been found in early foetal liver and have been termed carcinofoetal isoferritins (Alpert *et al.*, 1973). However, it has been proposed that these acidic carcinofoetal isoferritins are not specific for cancer or foetal tissue, but correspond to the acidic isoferritins in normal and adult heart (Arosio *et al.*, 1976).

The clinical implications of the release of these cancer-type isoferritins into the blood stream will be discussed in the following section.

1.5 Serum Ferritin

Ferritin was originally considered as an intracellular protein and was thought only to be present in serum in such grossly pathological states as acute liver damage, when release by necrotic cells might be expected (Reismann and Dietrich, 1956; Aungst, 1966). The development of a sensitive immunoradiometric assay for ferritin (Addison *et al.*, 1972) has shown that it is normally present in serum (Cook *et al.*, 1974; Jacobs *et al.*, 1972; Siimes *et al.*, 1974) and in circulating blood (Summers

et al., 1974).

The ferritin concentration in serum is directly related to the available storage iron in the body, the mean being higher in men than in women, with a range between 12 and 300 $\mu\text{g}/\text{l}$ (Cook *et al.*, 1974; Jacobs *et al.*, 1972; Jacobs and Worwood, 1975). The distribution does however vary from individual to individual and high concentrations may be found in subjects who are otherwise normal. In general, in patients with iron deficiency anaemia, serum ferritin concentrations are below 12 $\mu\text{g}/\text{l}$ while in patients with iron overload the concentration may be as high as 10 mg/l (Jacobs *et al.*, 1972; Cook *et al.*, 1974). Abnormally high levels have been shown in patients with acute liver disease (Prieto *et al.*, 1975), with the highest levels, up to 27 mg/l , being found in patients with viral and drug-induced hepatic necrosis. There is also a close correlation between serum ferritin concentration and mobilisable iron stores in haemochromatosis (Beamish *et al.*, 1974).

High serum ferritin concentrations inappropriate for the amount of storage iron, have also been found in patients with leukaemia (Jones *et al.*, 1973; Worwood *et al.*, 1974), with the highest concentrations in patients with acute myeloblastic leukaemia. The high concentration found in this condition appears to be related to an

increase in production of ferritin by leukaemic cells (Worwood *et al.*, 1974). High concentrations of serum ferritin have also been shown in Hodgkin's disease (Beiber and Beiber, 1973; Jones *et al.*, 1973).

Studies on the nature of serum ferritin suggest that it is normally closely related to reticuloendothelial ferritin. There is a close relationship between the two at all levels of iron storage from the deficient state to gross overload. When a shift in iron between the reticuloendothelial and red cell compartments occurs it is reflected by a corresponding change in the concentration of circulating ferritin, this effect is seen in the first month of life, after major venesection, in the anaemia of chronic disease, and during the treatment of pernicious anaemia with vitamin B₁₂ (Jacobs, 1976). This observation has been confirmed by experiments in rats in which the entry of ferritin into the plasma was studied after the injection of ⁵⁹Fe-labelled compounds (Siimes and Dallman, 1974). Brief labelling of serum ferritin was found 20-40 minutes after the injection of heat treated ⁵⁹Fe-labelled red cells, but not after ⁵⁹Fe-haemoglobin-haptoglobin, suggesting that plasma ferritin originates in the cells concerned with the breakdown of effete red cells.

Serum ferritin differs markedly from purified pre-

parations of ferritin extracted from several tissues (Worwood *et al.*, 1975). On anion exchange chromatography with DEAE-Sephadex A-50, serum ferritin has a lower affinity than many of the tissue ferritins examined. It also behaves similarly to apoferritin on sucrose density centrifugation, suggesting that it has a low iron content.

Isoelectric focusing studies on serum ferritin from a normal patient have shown it to have an isoferritin profile more basic than most tissue ferritins (Arosio *et al.* 1977). However, serum ferritin from a patient who suffered from haemochromatosis and died from a malignant hepatoma had several more acidic isoferritins, which appeared to correspond approximately to the more acidic isoferritins in ferritin purified from the tumour of malignant hepatoma (Powell *et al.*, 1975). Antisera has been raised against ferritin from cancer tissue which can distinguish immunologically, serum ferritin with isoferritins characteristic of cancer-type ferritin. It has been proposed that a radioimmunoassay specific for cancer-type serum ferritin could be used as a tumour marker (Hazard and Drysdale, 1977).

1.6 The Subunit Structure of Apoferritin

That apoferritin consisted of subunits and not one long polypeptide chain was first demonstrated by Hofmann

and Harrison (1963). They achieved a partial splitting of the protein into a 2.1 S component with dilute alkali, while at SDS-protein ratios higher than 1:3 (w/w) the product was all in the form of a 2.5 S protein-SDS complex. The molecular weight of the complex was obtained from both measurements of S and D (sedimentation and diffusion constants) measured separately, and measurements of the ratio S:D from the Archibald approach-to-equilibrium method. The apparent molecular weights showed a marked concentration dependence, caused by an increase of D and a decrease of S with concentration. The values extrapolated to infinite dilution gave a molecular weight of 38,000 to 41,000 for the complex and 25,000 to 27,000 for the protein subunit. Using these data, along with evidence from the number of peptides in tryptic digests of apoferritin (Harrison and Hofmann, 1963) and data from X-ray diffraction studies (Harrison, 1963), which showed that the quaternary structure of apoferritin might consist of 20 subunits at the apices of a pentagonal dodecahedron, Hofmann and Harrison (1963) concluded that apoferritin consisted of 20 identical subunits of molecular weight 24,000.

Subsequent to this work, two techniques were developed which could be used to measure with a good degree of accuracy the molecular weight, under dissociating conditions, of protein subunits:

- (a) gel filtration on columns of agarose equilibrated in 6 mol/l guanidine hydrochloride (Fish *et al.*, 1969)
- (b) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (Maizel, 1966).

Using these two new techniques, and sedimentation equilibrium under dissociating conditions in the analytical ultracentrifuge, Bryce and Crichton (1971) and Bjork and Fish (1971) reinvestigated the subunit molecular weight of apoferritin. Results obtained indicated that the polypeptide of horse spleen apoferritin had a molecular weight of 18,500. For reasons of symmetry, and because $24 \times 18,500$ comes much nearer the molecular weight measurements for apoferritin than $20 \times 18,500$, it was concluded that the apoferritin molecule consisted of 24 identical subunits of molecular weight 18,500.

The first indication that the subunits of apoferritin might not be identical came as a result of the identification on SDS electrophoresis and gel filtration in 6 mol/l guanidine hydrochloride of a small amount of lower molecular weight components (Bjork and Fish, 1971). The sum of the molecular weight of these smaller fragments approximated that of the 18,500 molecular weight component.

Niitsu *et al.* (1973) investigated the subunit structure

of liver ferritin from horse, human, rat and rabbit by PAGE in sodium dodecyl sulphate. Each ferritin preparation gave multiple protein staining bands on SDS electrophoresis. The molecular weights of the three major polypeptide chains were approximately 19,000; 15,000 and 10-11,000 while that of a fourth was estimated as being 7-8,000. The 15,000 molecular weight polypeptide was absent from human ferritin and was eliminated by treatment with β mercaptoethanol. On the basis of this observation, Niitsu *et al.* (1973) concluded that the 15,000 molecular weight polypeptide was not a native subunit, but was formed as a result of disulphide cross-linkages during the course of SDS denaturation of the protein. The four polypeptides were isolated by preparative gel electrophoresis in SDS, and the isolated components reexamined on analytical SDS gels. The two smallest components migrated as single species, while the 19,000 component was resolved into three components of molecular weight 19,000; 10-11,000 and 7-8,000. Repeated isolation and electrophoresis of the 19,000 component showed further breakdown to the 10-11,000 and 7-8,000 components.

Niitsu *et al.* (1973) concluded that although the multiple bands might originate from a proteolytic cleavage of a peptide occurring after isolation, it was also conceivable that they represented multiple subunit types.

A further report from the same laboratory (Ishitani *et al.*, 1975a) claimed that the 19,000 component was a composite of the 11,000 and 8,000 molecular weight polypeptides. This was based on the following evidence:

- (a) the sum of the amino acid composition of the two lower molecular weight species was almost identical to that of the 19,000 molecular weight species.
- (b) lysine amino-terminal residues were detected by dansylation for both the 19,000 and 11,000 molecular weight components of horse and rat ferritins.
- (c) when the 11,000 and 8,000 molecular weight polypeptides were mixed in equimolar amounts, they recombined to form substances that had identical electrophoretic mobilities as apoferritin, and also assumed the characteristic morphological appearance of apoferritin in electron micrographs.

Linder *et al.* (1974) were the first to report metabolic differences between the electrophoretic components. They identified on SDS electrophoresis, components of 13,000 and 19,000 molecular weight from rat liver and horse spleen apoferritin, which they claimed represented different apoferritin subunits. In the case of rat, the proportion of these varied with iron administration,

synthesis of the smaller "subunit" being preferentially stimulated by iron. This was consistent with an earlier report from the same laboratory that ferritin subunits of different molecular weights were synthesised on different polyribosome populations (Konijn *et al.*, 1973). Linder *et al.* (1974) concluded that the large subunit was preferentially synthesised on membrane bound polyribosomes of the liver, while the smaller subunit, whose synthesis was more readily stimulated by iron administration, was translated on the free ribosomes. These claims have recently been retracted (Zahringer *et al.*, 1977).

Messenger RNA from both bound and free ribosomes synthesised subunits with a molecular weight (SDS electrophoresis) of 19,000. The smaller ferritin "subunit" it was claimed was formed by scission of the original subunit either intracellularly or during isolation from the tissue (Zahringer *et al.*, 1974).

A further model for the quaternary structure of apoferritin was suggested by Adelman *et al.* (1975). When they analysed human heart and liver ferritin by acetic acid urea electrophoresis, both ferritins contained two protein staining components, one of which was common to both. The three bands were designated H, HL, and L, L being the most cathodic and H the least cathodic. Heart ferritin contained the H and HL components while liver ferritin contained the HL and L. Reduction with dithio-

threitol eliminated the L band indicating that it was possibly artifactual, caused as a result of disulphide cross-linking (Adelman *et al.*, 1975).

The H and HL components were originally thought to have similar molecular weights (Adelman *et al.*, 1975), but experiments using two-dimensional electrophoresis (acetic acid urea in the first-dimension, and SDS gradient pore in the second) indicated that a molecular weight difference did exist (Drysdale *et al.*, 1977). Molecular weights of 21,000; 19,000 and 15,000 were assigned to the H, and HL and the unreduced L component. Components of molecular weights similar to those assigned to H, HL and L have also been observed on SDS gradient pore gels (Drysdale *et al.*, 1977).

Drysdale *et al.* (1975) have proposed that the H and HL polypeptides are the structural basis for isoferritins. Ferritin they regard as a family of hybrid molecules fashioned from these polypeptide chains. The relative proportions of the two subunits in different ferritins varies with their pI range. The acidic isoferritins such as those in heart contain up to 70% of the H subunit and 30% of the HL subunit, whereas the basic isoferritins of liver or spleen contain less than 30% H and at least 70% HL.

Table 1 summarises the results obtained by various

groups of workers studying the subunit structure of apo-ferritin.

Table 1. Results Reported for the Subunit Structure of Apoferritin

| | Method of Dissociation | Method of Analysis | Subunit m.wt. |
|---|-------------------------------------|--|--|
| Hofmann & Harrison (1963) | SDS | I. Equilibrium Dialysis II. Ultracentrifugation | 24,000 1 |
| Bryce & Crichton (1971) Bjork & Fish (1971) | SDS Guanidine HCl Acetic Acid | I. SDS Electrophoresis II. Gel Filtration III. Ultracentrifugation | 18,500 1 |
| Niitsu <i>et al.</i> (1973) Ishitani <i>et al.</i> (1975a) Ishitani <i>et al.</i> (1975b) | SDS | SDS Electrophoresis | 19,000 1, 2, 3, 4 15,000 10-11,000 7- 8,000 |
| Linder <i>et al.</i> (1974) | SDS | SDS Electrophoresis | 19,000 1, 2 13,000 |
| Adelman <i>et al.</i> (1975) Ishitani <i>et al.</i> (1975b) Drysdale <i>et al.</i> (1977) | Acetic Acid | Acetic Acid Urea Electrophoresis | 21,000 1, 4 19,000 15,000 |
| Drysdale <i>et al.</i> (1977) | SDS | Gradient SDS Electrophoresis | 21,000 1, 4 19,000 15,000 |

¹Horse Spleen ³Rabbit Liver

²Rat Liver ⁴Human Liver & Heart

CHAPTER II

THE PRESENT PROBLEM

It can be deduced from the publications summarised in Table 1 that there remain three possibilities for the general quaternary structure of apoferritin; that the molecule consists simply of 24 identical subunits, that it consists of unspecified numbers of two or three subunits of which one or two have a molecular weight substantially less than the 18,500 proposed in the unitary hypothesis, and that it consists of varying proportions of two subunits of similar but not identical mass, the total probably 24. The second of these hypotheses admittedly carries less weight since the recent retraction by Munro's laboratory of some of its earlier findings (Zahringer *et al.*, 1977; Linder *et al.*, 1974), but that is still a highly controversial area.

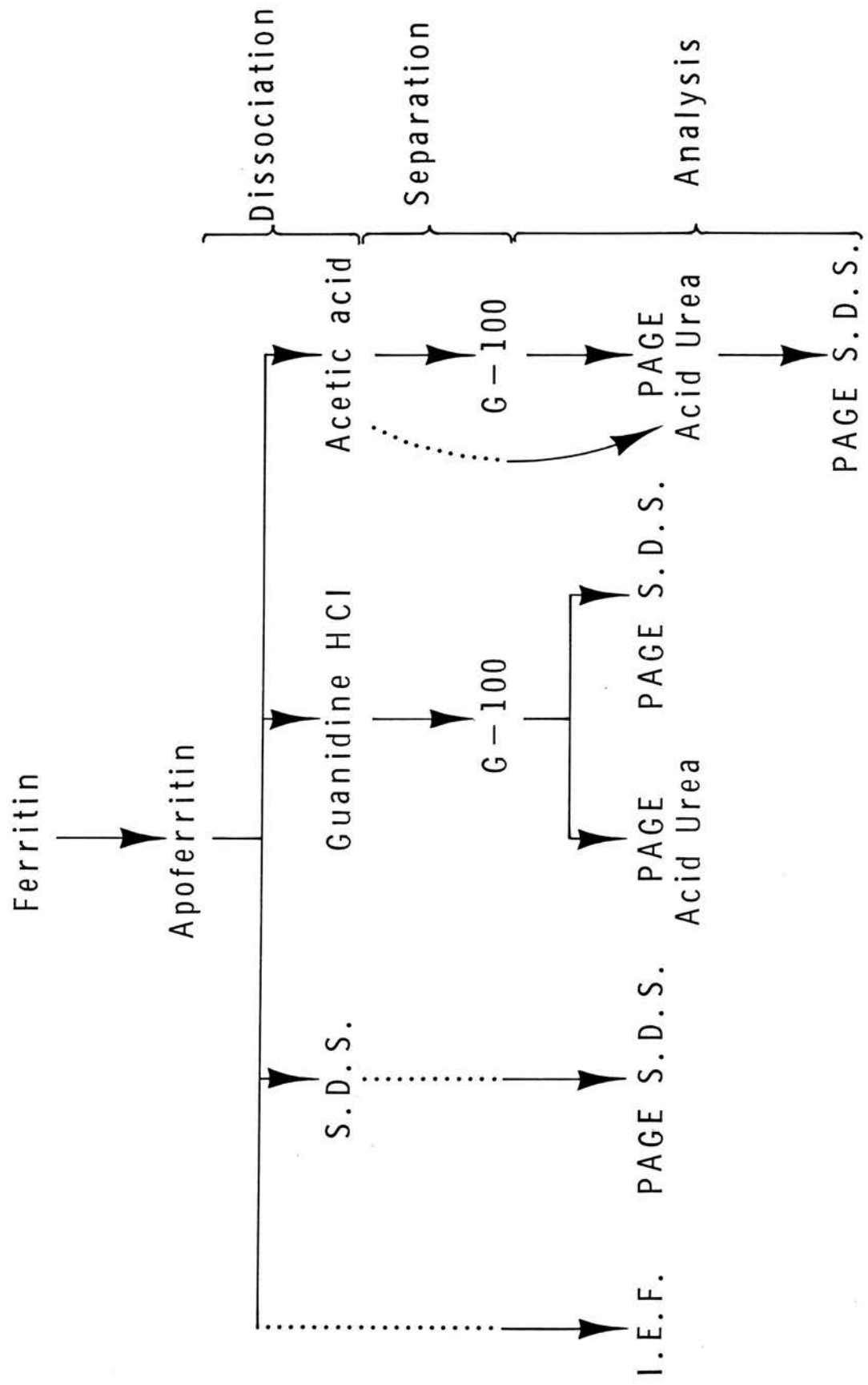
The whole subject not only has inherent biological interest but has practical importance for the study of iron metabolism and also in connection with possible differences in antigenic specificity in the radioimmunoassay of serum ferritin. The work described in this thesis was undertaken in the hope of obtaining information which might help to reconcile what appear to be inconsistencies in the work discussed in the preceding pages. It was planned:

1. To study apoferritin prepared from three different ferritins: horse spleen, because it is the subject

of most published structural investigation; rat liver, because it is the ferritin most commonly studied in model metabolic experiments; and human spleen, because of the growing interest in serum ferritin levels in various disease conditions.

2. To prepare ferritin by three different methods in order to test the hypothesis that the multiple bands produced by SDS electrophoresis in most laboratories are an artefact of one or more stages in the isolation.
3. To dissociate apoferritin from each of the three sources and analyse the products by methods as far as possible identical with those used by the original workers:
 - (i) dissociation in 67% acetic acid, 7 mol/l guanidine hydrochloride and SDS.
 - (ii) analysis by gel filtration on sephadex G-100 in the appropriate buffer system, and by SDS electrophoresis and acetic acid urea electrophoresis.
4. To perform cross-check experiments in which the products of one type of dissociation or analysis will be subjected to a different analytical proce-

Fig.1: Scheme of the General Approach to the Problem.



ture. This would involve the following experiments:

- (i) subjecting the products obtained from dissociation in guanidine hydrochloride to acetic acid urea electrophoresis and SDS electrophoresis.
- (ii) subjecting the chromatographically separated acetic acid dissociated products to acetic acid urea electrophoresis
- (iii) subjecting the acetic acid urea electrophoretic products to SDS electrophoresis.

A scheme of this approach is outlined in Fig. 1.

A brief word at this point may be helpful to the reader in understanding the further layout of this thesis. Chapter three will describe the tissue source, purification, and to an extent the characterisation of the material to be studied. The following two chapters will then deal with the dissociation, chromatographic separation (Chapter 4), and electrophoretic analysis (Chapter 5), while Chapter 6 will cover the cross-check experiments. The author has decided to abandon the usual pattern which involves collecting and segregating all methods in a special chapter. Instead the methods dealing with specific analyses will be described in the relevant

chapters. It is felt that the layout adopted makes the thesis more readable and the proximity of the methods to the relevant results facilitates the understanding of some rather complicated experiments.

CHAPTER III

PREPARATION OF FERRITIN AND ANALYSIS

• OF THE ISOLATED PRODUCT

BY ISOELECTRIC FOCUSING

3.1 Introduction

This chapter describes the source of tissues used in these studies, the methods used to isolate ferritin, prepare apoferritin, to check on the purity of the preparations, and to analyse the isolated products by isoelectric focusing.

3.2 Source of Tissues

The horse spleens were obtained from the Pathology Department, Veterinary Field Station, University of Edinburgh. The spleens were taken from slaughtered horses immediately after death, cut into small portions and placed in a deep freeze at -40°C .

Rats of the Wistar strain were obtained from the Small Animal Unit, Veterinary Field Station, University of Edinburgh. In all experiments, the rats were female, since female rats have a higher liver ferritin content than male rats (Bjorklid and Helgeland, 1970; Stanton and Ramsay, 1975). On removal from the animal, the liver was quickly frozen by placing the tissue under a pre-chilled metal block in the freezing compartment of a refrigerator.

The human spleens were obtained through the Department of Haematology, Royal Infirmary, Edinburgh. All those used were from patients treated by splenectomy for

haemolytic conditions. The tissue was chilled in the operating theatre, brought to the laboratory within one or two hours, and deep frozen.

All tissues were stored in a deep freeze at -40°C .

3.3. Procedures Used in the Isolation of Ferritin

The original procedure developed by Laufberger (1937) and Granick (1924) took advantage of the stability of ferritin to heating at 80°C , its precipitation by half saturation with ammonium sulphate, and its ready crystallisation from 5% cadmium sulphate.

A number of modifications to this isolation procedure have been used. Drysdale and Ramsay (1965) used C.M.-cellulose to separate ferritin from other iron containing proteins, while Drysdale and Munro (1966) employed these observations to develop an isolation procedure, which involved chromatographing a heat supernatant (70°C) on C.M.-cellulose, followed by ammonium sulphate precipitation and Sephadex G-200 chromatography. A method for the isolation of ferritin utilising its high density to purify it by high speed centrifugation was described by Penders *et al.* (1968). Bjorklid and Helgeland (1970), on the other hand developed a method involving polyacrylamide gel electrophoresis for the small scale isolation of ferritin.

The isolation of delicately structured molecules from complex mixtures always carries the risk of incidental destruction or damage, and for many years there have been doubts about the heat coagulation step in the isolation of ferritin. Many workers prefer 70°C to the original 80°C, and Yu and Fineberg (1965) found that the yield in an isolation procedure involving DEAE-cellulose was improved if the temperature during the preliminary heat coagulation did not exceed 65°C. Stanton (1975) found that the preliminary heat coagulation diminished the recovery of ferritin iron from cm-cellulose, and that this effect became worse as the temperature was increased. His solution was to defer heat coagulation until after completion of the C.M-cellulose step. Crichton (Collett-Cassart and Crichton, 1975) suggested that heat coagulation of the crude homogenate might allow proteolytic damage to occur. Crichton (personal communication) proposed that this could be overcome by carrying out the heat coagulation in small volumes of the homogenate, so that the increase in temperature to 70°C was as rapid as possible, thus avoiding subjecting the homogenate to lengthy intermediate temperatures, during which proteolytic activity could occur.

Three isolation procedures were used in the work described in this thesis. In two of these procedures one major and potentially harmful step was omitted. The

first of these was that of Stanton (1975) involving both ion exchange chromatography and heat coagulation at 70°C. The second was that used in the Department of Haematology, Welsh National School of Medicine, Cardiff (A. Jacobs, personal communication), which avoids the use of ion exchange chromatography, and the third was a composite of these two which omitted heat coagulation at 70°C.

Isolation Procedure 1 : Involving Ion Exchange Chromatography and Heat Treatment (Stanton 1975)

The tissue (approx. 50 g) was homogenised, 1 part tissue to 4 parts water in a top drive homogeniser (MSE). The homogenate was brought to pH 4.8 by addition of 0.5 mol/l acetic acid, and centrifuged at 7,000 RPM (MSE 18 centrifuge) for 20 minutes. The precipitate was washed twice with 0.025 mol/l acetate buffer pH 4.8, centrifuging at 7,000 RPM for 15 minutes after each washing. The supernatant and washings were combined, and diluted to twice the volume of the original homogenate. This diluted supernatant was subjected to ion exchange chromatography on a battery of C.M-cellulose columns (3.2 cm diameter × 8 cm length), previously equilibrated with acetate buffer pH 4.8 (0.025 mol/l). The pH 4.8 supernatant was applied to the columns (maximum volume, 60 ml per column), and washed on with acetate buffer pH 4.8 (0.025 mol/l). During the preparation of rat liver

ferritin, a turbid eluate appeared at this stage. Washing with pH 4.8 buffer was continued until the turbidity of the eluate cleared. With horse and human spleen, no turbidity was observed. Ferritin was eluted by passing acetate buffer pH 5.5 (0.1 mol/l) through the column. The progress of ferritin elution could be observed as a dark brown band moved down the column. All operations up to this point were performed at 4°C.

The ferritin eluate (approximately 200 ml) was divided into volumes of about 50 ml, heated in a water bath at 70°C, and maintained at this temperature for ten minutes before cooling in ice. The coagulated material was removed by centrifugation at 7,000 RPM for 15 minutes.

Ferritin was precipitated, 4°C for 15 hours, from the heat supernatant by ammonium sulphate added as solid in sufficient quantity to give a final saturation of 60% (36.1 g/100 ml). The precipitated protein was recovered by centrifugation at 7,000 RPM for 30 minutes, and dissolved in 2.0 ml of a solution containing 0.014 mol/l NaCl and 0.002 mol/l sodium bicarbonate.

The dissolved protein was subjected to a final purification involving gel filtration on Sephadex G-200. The column (90 cm × 2 cm²) was equilibrated in either phosphate buffer pH 7.0 (0.02 mol/l) or a borate buffer pH 8.5 containing 0.05 mol/l boric acid, 0.01 mol/l

disodium tetraborate and 0.1 mol/l sodium chloride. The column flow rate was 2 ml/h, and the ferritin fractions could be detected visually by the elution of a brown solution.

Isolation Procedure 2 : Involving Heat Coagulation and Ultracentrifugation

This procedure is based on a personal communication from A. Jacobs and described by Worwood *et al.* (1975).

The tissue (approximately 50 g) was homogenised, 1 part tissue to 4 parts water in a top drive homogeniser (MSE). The homogenate was heated in a water bath at 70°C, and was maintained at this temperature for 10 minutes before cooling on ice. After centrifugation at 7,000 RPM for 15 minutes to remove heat precipitated material, the supernatant was adjusted to pH 4.8 with acetic acid (1 mol/l). The precipitate formed at pH 4.8 was removed by centrifugation at 7,000 RPM for 15 minutes, and the pH of the supernatant adjusted to 5.5 with acetic acid (1 mol/l), after solid sodium acetate had been added to a concentration of 0.05 mol/l.

Ferritin was precipitated, 4°C for 15 hours, from this solution by the addition of solid ammonium sulphate in sufficient quantity to give a final saturation of 60%, and the precipitated protein recovered by centrifugation

at 7,000 RPM for 20 minutes.

The precipitated protein was dissolved in approximately 10 ml of a salt bicarbonate solution containing 0.014 mol/l sodium chloride and 0.002 mol/l sodium bicarbonate, and centrifuged at 5,000 RPM for 15 minutes. The supernatant was then centrifuged at 100,000 g (Omikron ultracentrifuge) for 2 hours, and the pellet obtained dissolved in 2 ml of the salt bicarbonate solution.

The ferritin solution was finally subjected to gel filtration on Sephadex G-200 (column size 90 cm \times 2 cm²) equilibrated in phosphate buffer pH 7.0 (0.02 mol/l) or borate buffer pH 8.5 (0.06 mol/l) containing 0.1 mol/l sodium chloride.

Isolation Procedure 3 : Involving Ion Exchange Chromatography and Ultracentrifugation

This procedure is derived from the two methods described above, and was designed to avoid the use of a heat treatment stage.

A 1:4 tissue homogenate was brought to pH 4.8 by addition of 1 mol/l acetic acid, the precipitated material removed by centrifugation at 7,000 RPM for 20 minutes, and the pH 4.8 supernatant subjected to ion

exchange chromatography on columns (3.2 cm diameter \times 8 cm long) of C.M-cellulose equilibrated in acetate buffer pH 4.8 (0.025 mol/l). The ferritin was eluted by addition of acetate buffer pH 5.5 (0.1 mol/l).

The heat treatment was omitted at this stage and the ferritin was precipitated, 4°C for 15 hours, by addition of solid ammonium sulphate to give a final saturation of 60%. The precipitated protein was collected by centrifugation at 7,000 RPM for 15 minutes, dissolved in 10 ml salt bicarbonate solution and centrifuged at 100,000 g for 2 hours (Omikron ultracentrifuge). The pellet obtained was dissolved in 2 ml of the salt bicarbonate solution and subjected to gel filtration on sephadex G-200 as previously described. The ultracentrifugation and gel filtration steps were repeated, where necessary, to obtain a pure ferritin preparation.

3.4 Check on the Purity of Ferritin Preparations

The purity of the ferritin preparations was tested by polyacrylamide gel electrophoresis in 5% polyacrylamide gels, which were stained for both iron and protein, and by SDS polyacrylamide gel electrophoresis. Routinely the latter electrophoretic system was used, since sometimes preparations which appeared pure on 5% polyacrylamide gels had multiple impurity bands on SDS electrophoresis. These bands were faint, apparently corresponding

to molecular weights substantially higher than 21,000. That they were also observed when the analysis was made after treatment of the specimen with DTT showed that they were not disulphide linked oligomers of subunits. That they did represent impurities was proved by the fact that they were not found after the preparation had been rechromatographed on Sephadex G-200.

PAGE in 5% Polyacrylamide Gels

Electrophoresis was carried out in glass tubes (10 cm × 0.6 cm). The gels were prepared from a stock solution, which contained 5 g acrylamide in 100 ml of tris glycine buffer pH 9.3, prepared by adjusting the pH of 0.05 mol/l tris to 9.3 by the addition of glycine. Gel polymerisation was achieved by the addition to 25 ml of this stock solution, of 0.25 ml 7% (w/v) ammonium persulphate and 0.015 ml Temed. The electrophoresis buffer was tris glycine pH 9.3 (0.05 mol/l). Prior to electrophoresis, the gels were pre-run for 45 minutes at 5 mA/gel, while the ferritin samples were then subjected to electrophoresis at 3 mA/gel for two hours.

The gels were stained for protein by immersing them in 0.1% (w/v) naphthalene black in 7% (v/v) glacial acetic acid for two hours, and destained in acetic acid (7% v/v). The Prussian Blue reaction was used to stain

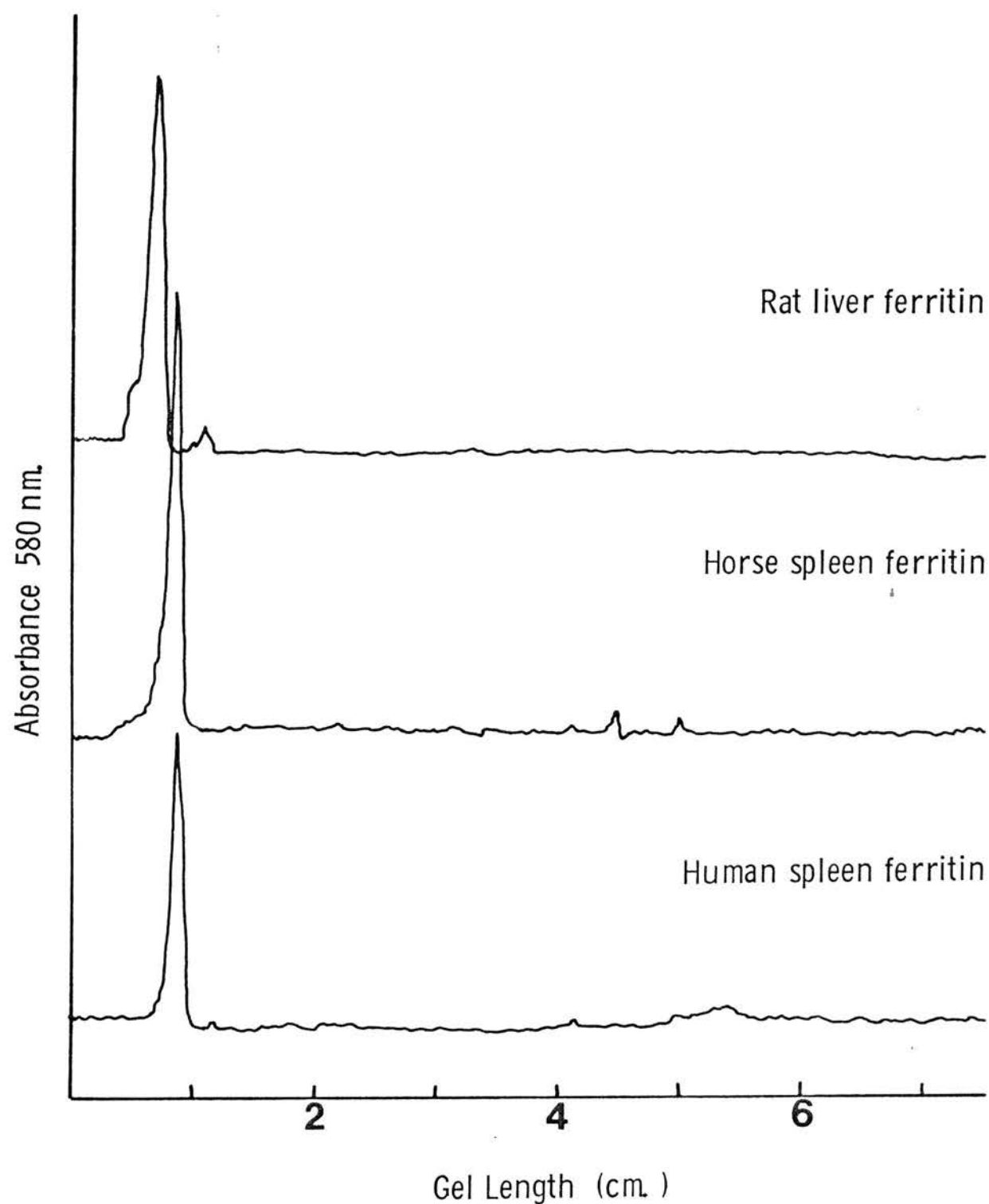


Fig. 2 Densitometric scans of protein stained 5% polyacrylamide gels of :

- 1) human spleen ferritin
- 2) horse spleen ferritin
- 3) rat liver ferritin.

The ferritin samples were all prepared by method 1), which involved heat treatment and cm-cellulose chromatography.

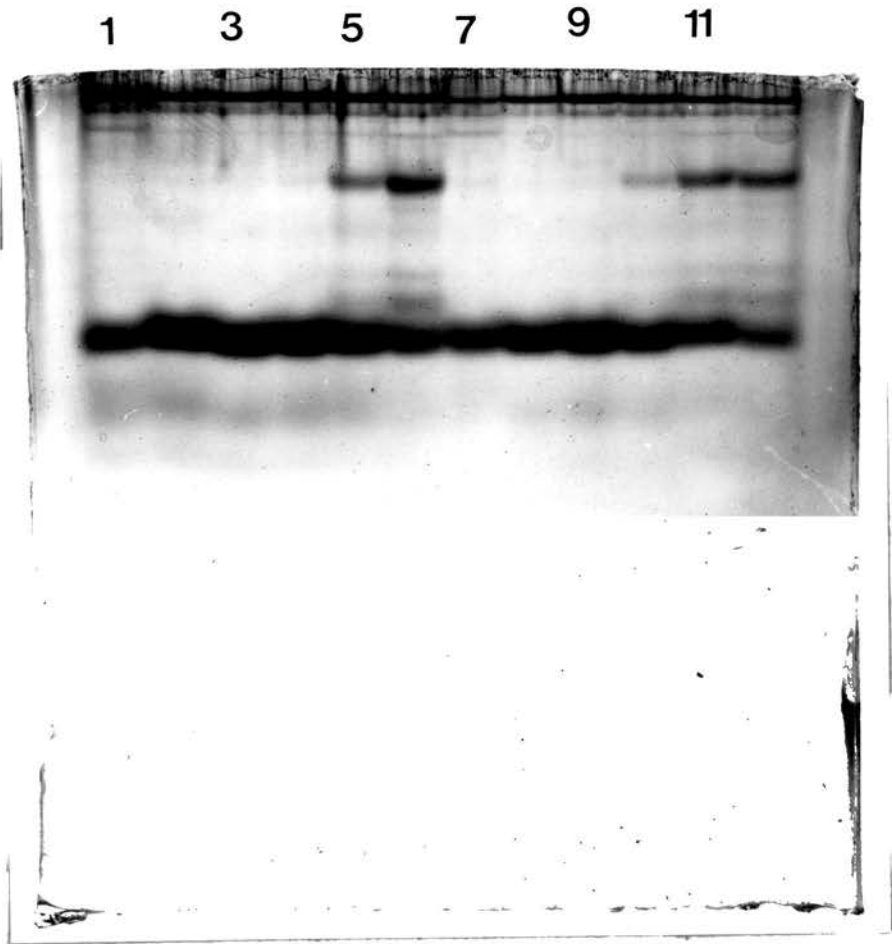


Fig. 3 SDS 10% polyacrylamide slab gel after electrophoresis of ferritin fractions eluted from Sephadex G-200 in the final purification stage of the isolation procedure. Samples applied to the were : nos. 1-6 horse spleen ferritin, and nos. 7-12 human spleen ferritin.

for iron, using potassium ferrocyanide (2% w/v) in hydrochloric acid (2% v/v) (Linder-Horowitz *et al.*, 1970).

Gels stained for protein were scanned at 580 nm (Section 5.3). Scans of the 5% polyacrylamide gels of human spleen, horse spleen and rat liver isolated by method (i) are shown in Fig. 2. Scans identical to these were obtained for preparations isolated by methods (ii) and (iii). In all ferritin preparations, two iron and protein staining bands were observed: a major band representing the monomeric species and a slower migrating, fainter staining band (not obvious on the scans in Fig. 2), which was assumed to represent a ferritin dimer. The purity of the ferritins isolated by all three methods was based on the sole presence of these two iron and protein staining bands, (cf. limitations described above).

PAGE in the Presence of SDS

The preparation and use of SDS gels is described in Chapter 5 (Section 5.3). An SDS 10% polyacrylamide slab gel of horse spleen and human spleen ferritin fractions eluted from Sephadex G-200 is shown in Fig. 3. Horse spleen ferritin fractions 1-4 and human spleen ferritin fractions 7-9 show only one main protein staining band, along with the two typical fainter staining lower molecular weight bands (these will be discussed at greater length in

Section 5.3). Fractions 5 and 6, and 10, 11 and 12 show a number of protein staining bands above the main band which represent impurities, on the criteria described above. Only the fractions shown by this criterion to be pure were pooled in the final preparation. Routinely it was found that only the last two or three fractions out of a total of approximately 10×2 ml fractions showed impurities.

In general, the three procedures described for the isolation of ferritin produced preparations which behaved almost identically on the two electrophoretic techniques used as a basis to establish purity. One significant difference however was that the rat liver preparations, regardless of the method of isolation, were less mobile on 5% PAGE than either of the two spleen ferritins. This could be due to the rat liver ferritin having a larger size or having a more positive net charge than either of the spleen ferritins.

Although the isolation procedures adopted had no apparent effect on the electrophoretic properties of the whole molecule, the object of employing three different methods was to determine the effect of the various stages employed in the isolation on the subunit structure.

3.5 Preparation of Apoferritin

The original method for the preparation of apoferritin (Granick, 1942) involved dialysis of the ferritin against 1% (w/v) sodium dithionite and 1% (w/v) α, α' dipyridyl at pH 4.6. Autoxidation of dithionite however may give colloidal sulphur which contaminates the apoferritin (Behrens and Taubert, 1952; Crichton, 1969). This can be overcome by carrying out the dialysis under nitrogen gas in the cold at pH 5.0 - 5.2. The iron dipyridyl complex can be removed by repeated dialysis against distilled water or by gel filtration on Sephadex G-25 (Suran and Tarver, 1965).

Native apoferritin can be prepared by density gradient centrifugation in isopycnic gradients of CsCl or sucrose (Fishbach and Anderegg, 1965) and although fractionation according to iron content by ammonium sulphate has also been reported (Philipott and de Bornier, 1959), this procedure does not seem to be suitable for the preparation of iron-free apoferritin.

The method employed in these studies (Crichton, personal communication), involves dialysis of the ferritin preparation against 1% (w/v) mercaptoacetic acid. This method was selected since there is no problem of sulphur precipitation, and the time taken to remove all the iron is typically less than 6 hours. Once the ferritin solu-

tion becomes colourless, the apoferritin is dialysed against several changes of distilled water. All apoferritin samples were lyophilised and stored at -40°C .

During the preparation of apoferritin by this method, the pH of the ferritin solution falls to about 2, under which circumstances the apoferritin dissociates into its subunits. When the preparation is then dialysed against distilled water, the pH increases and the subunits reassociate to form whole apoferritin (Crichton and Bryce, 1973). This whole apoferritin did not behave differently on 5% PAGE or SDS PAGE from apoferritin prepared by a method whereby the mercaptoacetic acid was buffered at pH 5.2.

3.6 Isoelectric Focusing of Ferritin and Apoferritin

Introduction

Isoelectric focusing is an equilibrium method in which amphoteric molecules are segregated according to their isoelectric points in pH gradients. The pH gradients are formed by electrolysis of amphoteric buffer substances known as ampholytes. When introduced into this system, other amphoteric molecules such as proteins will migrate to pH zones that will correspond to their respective pI's, where their net charge is zero. The technique has now been refined to a level that permits

the resolution of molecules whose pI's differ by as little as 0.005 pH unit or less.

The development of IEF can be traced to the pioneering work of Ikeda and Suzuki (1912), who found that a mixture of amino acids would assume an order during electrolysis that followed the ascending pI values between the anode and the cathode. The technique of IEF received its stimulation during 1954-56 (Kolin, 1954; 1955) with the development of a series of pH gradients using diffusion of buffers in sucrose density gradients under an electric field. Unfortunately these gradients were unstable because of the rapid migration of the buffering electrolytes during electrolysis, and the recovery of separated components proved difficult.

The concept of IEF was extended in the 1960's by Svensson (Svensson, 1961; 1962a; 1962b), who advanced the idea of developing natural pH gradients from amphoteric molecules with high conductances and closely spaced pI's. Vesterberg (1969) synthesised carrier ampholytes with many of the properties described by Svensson. With these ampholytes, it became possible to develop smooth and stable pH gradients between 3 and 10.

Initially, IEF was conducted in sucrose density gradients on an anticonvective medium. However, IEF in polyacrylamide gels became popular, since it overcame

many of the problems associated with convective mixing; diffusion and isoelectric precipitation in liquid media. Early developments have been reviewed by Catsimpoolas (1970; 1973), Williamson (1973) and Wrigley (1971).

This section describes thin layer electrofocusing in polyacrylamide gels of horse spleen, human spleen and rat liver ferritins and apoferritins.

Methods

Preparation of gels

Stock solutions:

- A. Acrylamide 29.1% (w/v) in distilled water.
- B. N, N methylenebisacrylamide 0.9% (w/v) in distilled water.

These two solutions were filtered through Millipore filters to remove any particles.

- C. Riboflavin 0.008% (w/v).
- D. Ammonium persulphate 1% (w/v).

All stock solutions were stored in dark bottles at 4°C. A, B and C were used within two weeks and D within one week after preparation.

To prepare gels in the pH range 3.5 to 9.5, the following solutions were mixed:

10.0 ml A

10.0 ml B

36.0 ml distilled water in which 7.5 g sucrose was dissolved

2.8 ml Ampholine pH 3.5 - 10

0.4 ml Ampholine pH 9.0 - 11

0.2 ml Ampholine pH 4.0 - 6

0.2 ml Ampholine pH 5.0 - 7

To prepare gels in the pH range 4 to 6, the following solutions were mixed:

10 ml A

10 ml B

38.4 ml distilled water in which 7.5 g sucrose was dissolved

1.2 ml Ampholine pH 4 - 6

0.5 ml Ampholine pH 3.5 - 10


In both preparations, the solutions were mixed thoroughly and deaerated by aspirating for one or two minutes. Finally 0.4 ml solution C and 0.8 ml solution D were added.

Gel Polymerisation

The glass plates (24 cm × 10 cm) were washed in lukewarm water. A final washing in distilled water and ethanol was carried out before the glass plates were allowed to dry. The unpolymerised polyacrylamide gel solution was introduced by means of a pipette between the two glass plates separated by a 1 mm thick rubber gasket. Photopolymerisation using riboflavin as catalyst occurred by placing the plates above a daylight photopolymerisation lamp.

Temed was not used in this system, because the carrier ampholytes contain enough tertiary amino groups to catalyse the polymerisation reaction, while sucrose was present within the gel solution as it improves the reproducibility of the gel polymerisation without having any effect on the pore size of the polyacrylamide gel. The sucrose also has a positive effect on the stability of the established pH gradient since it increases the viscosity in the gel.

The photopolymerisation was allowed to continue for one hour, whereupon the polymerisation set was placed in the refrigerator at 4°C for ten to fifteen minutes, during which period the polyacrylamide gel becomes more stable and the upper glass plate can be easily removed. A thin narrow spatula was used to remove the 3 mm thick



upper glass plate from the 1 mm thick lower glass plate on which the polyacrylamide gel was intended to stay.

Sample Application

The samples were applied using Whatman Chromatography Paper 3 mm cut into 5 × 10 mm pieces. These were placed on the surface of the gel, close to the cathode and approximately 15 to 20 μ l of sample was applied to each paper. The sample was always applied before formation of the pH gradient.

Electrofocusing

The isoelectricfocusing was carried out on the LKB Multiphor apparatus. The electrode strips were soaked in the electrode solutions which were 1 mol/l H_3PO_4 for the anode and 1 mol/l NaOH for the cathode. Electro-focusing occurred over a period of three hours, during which time the current dropped from 30 mA to 6 mA and the voltage increased from 600 v to 1200 v. After thirty minutes, the power was switched off, and the filter papers removed from the surface of the gel.

Once the electrofocusing was finished, the pH gradient was determined using a surface pH electrode.

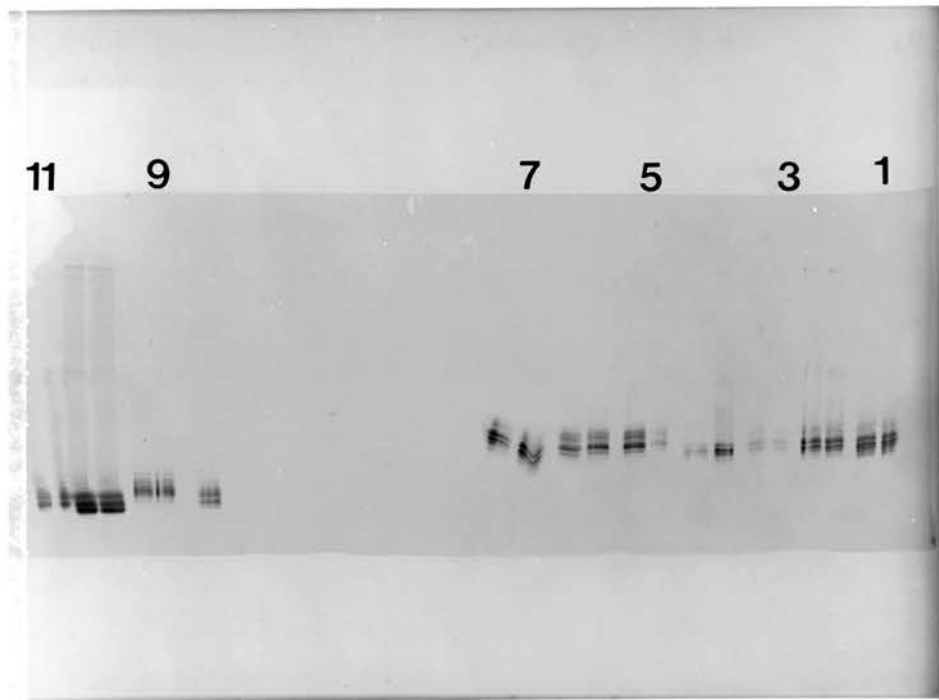


Fig. 4 Isoelectric focusing on a pH gradient 3.5-9.5 of (in duplicate):

- 1) rat liver ferritin (method 1)
- 2) and 3) rat liver ferritin (method 2)
- 4) rat liver apoferritin (method 1)
- 5) human spleen ferritin (method 1)
- 6) human spleen ferritin (method 2)
- 7) human spleen apoferritin (method 1)
- 8) horse spleen ferritin (method 1) - single sample
- 9) horse spleen ferritin (method 2)
- 10) horse spleen ferritin (Miles Pentex)
- 11) horse spleen apoferritin (method 1).

Staining

The stain (Malik and Berrie, 1972) was prepared as follows: 2 g Coomassie BrilliantBlue G-250 (Sigma) was dissolved in 100 ml distilled water, 100 ml 1 mol/l H_2SO_4 was added, and the solution stirred for one hour. The precipitate was allowed to sediment overnight and the solution was filtered through Whatman Number 1 filter paper. Approximately 7 ml potassium hydroxide (10 mol/l) was added to the red solution until it turned blue. This solution was made up to 400 ml with a perchloric acid solution such that the final concentration was 3.5% with respect to perchloric acid, and filtered through Whatman Number 1 paper.

In this solution, staining of the protein was complete in about 30 minutes. No destaining was required.

3.6 Results

Isoelectricfocusing in a pH gradient 3.5 - 9.5 of a number of ferritin, and corresponding apoferritin preparations, isolated by methods 1 and 2 from horse spleen, human spleen and rat liver is shown in Fig. 4. Each preparation was resolved into several isoferritins with isoelectric points within the range pH 4.4 - 5.5. There was considerable overlap in banding profiles between ferritins and apoferritins from a single tissue. The acidity of the

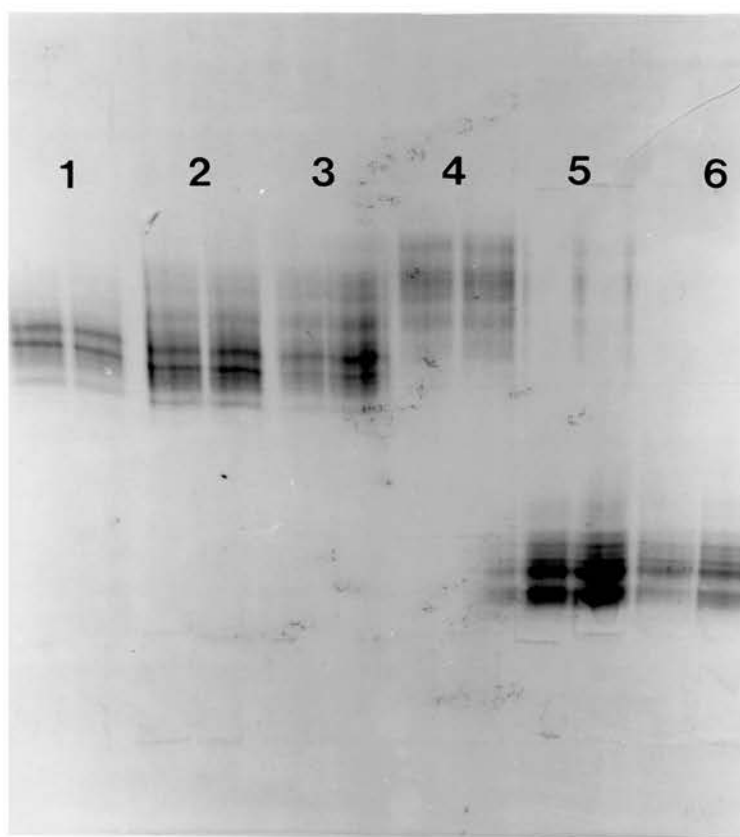


Fig. 5 Isoelectric focusing on a pH gradient 4-6 of :
1) rat liver ferritin (method 1)
2) rat liver ferritin (method 2)
3) rat liver apoferritin (method 1)
4) human spleen ferritin (method 1)
5) horse spleen ferritin (method 1)
6) horse spleen apoferritin (method 2).

isoferritins followed the pattern, horse spleen > rat > liver > human spleen. This is more clearly demonstrated in Fig. 5, which shows an isoelectric focusing gel within the pH range 4-6. The isoferritins were better resolved in this second gel, and it is possible to count at least 8 human spleen isoferritins. These were slightly more basic than the rat liver isoferritins, although as many as four were common to both proteins. The horse spleen isoferritins, on the other hand were more acidic than those from rat liver and human spleen. Seven isoferritins of horse spleen isoelectric points between pH 4.4 and 4.8 were noticeable.

By way of comparison, sample No. 10 on the gel shown in Fig. 4 was a commercial horse spleen ferritin preparation (6 x crystallised) purchased from Miles Pentex. This preparation had several more acidic isoferritins than those preparations isolated in this laboratory. It also contained a basic component, not obvious in any other preparation, which was presumed to be an impurity.

Insufficient evidence in the literature precludes the characterisation of a tissue ferritin on the sole basis of its isoelectric focusing profile. However, these analyses have demonstrated that there appears to be no charge differences between the ferritins isolated by methods 1 and 2. Unfortunately, no material prepared by method 3 was available when these results were obtained.

CHAPTER IV

DISSOCIATION OF APOFERRITIN AND SEPARATION OF THE DISSOCIATED PRODUCTS BY GEL FILTRATION

4.1 Introduction

Ferritin is comparatively stable in denaturants such as urea, organic solvents and detergents (Listowsky *et al.*, 1967). Several methods have however been successfully used to dissociate apoferritin into its constituent polypeptide chains: 67% acetic acid (Harrison and Hofmann, 1962), guanidine hydrochloride (Listowsky *et al.*, 1967), SDS (Hofmann and Harrison, 1963) and extremes of pH (Crichton and Bryce, 1973). This chapter describes gel filtration of the products obtained from apoferritin by dissociation with guanidine hydrochloride and 67% acetic acid. Niitsu *et al.* (1973) have pointed out that it was not possible to separate the products of dissociation of apoferritin in SDS by gel filtration in the presence of SDS.

Dissociation of any protein into its subunits will ordinarily result in an increase in the surface area of contact between protein and solvent, so that more groups are exposed to the solvent in the subunits than in the native molecule. Dissociation in high concentrations of acetic acid or urea, is favoured if most of the newly exposed groups are hydrophobic groups (Nazaki and Tanford, 1963). Guanidine hydrochloride, on the other hand, causes dissociation no matter what kind of new groups are exposed (Robinson and Jencks, 1963). In high concentrations of guanidine hydrochloride, proteins have been shown

to possess no residual non-covalent structure, and if all existing disulphide bonds are reduced, the protein polypeptide chains behave hydrodynamically as randomly coiled linear homopolymers (Tanford, 1968).

The radius of gyration (R_G) of these linear random coils is directly proportional to the Stokes radius, and is related to the molecular weight by the expression.

$$R_G^2 = \alpha^2 \beta^2 / 6 M_0$$

where α is the empirical factor which expresses the expansion of chain dimensions due to thermodynamic non-ideality and is a slowly increasing function of molecular weight. β is a measure of the effective monomer unit length and M_0 is the subunit molecular weight. When all the constant terms are collected, this equation becomes

$$R_G^2 = \text{constant } \alpha^2 M.$$

As a result of this relationship, gel filtration of proteins in high concentrations of guanidine hydrochloride has been used to determine with a high degree of accuracy the molecular weight of proteins and protein subunits (Fish *et al.*, 1969).

4.2 Methods

Dissociation of Apoferritin by Guanidine Hydrochloride

The apoferritin sample (approximately 10 mg) was dissolved in 0.3 ml of 7 mol/l guanidine hydrochloride containing 0.15 mol/l tris, 0.002 mol/l EDTA, and brought to pH 8.5 by the addition of hydrochloric acid. This solution was incubated at 37°C for at least 6 hours, 3 μ l β mercaptoethanol was added to reduce the disulphide bonds, and the solution was left under nitrogen gas for 12 hours at 4°C. (Listowsky *et al.*, 1967; Bryce and Crichton, 1971; Bjork and Fish, 1971).

In the majority of experiments, in order to avoid the formation of disulphide linkages, the sulfhydryl groups were protected by a carboxymethyl group. However in some experiments, the dissociated material was subjected to gel filtration without prior carboxymethylation. These experiments were specifically those in which the dissociated material was analysed by acetic acid urea electrophoresis, a technique which separates proteins on the basis of their charge to mass ratio. Consequently the charge on the carboxymethyl group would be likely to affect the properties of the dissociated material in this analysis.

For carboxymethylation, 0.2 ml of a solution containing 180 μ moles iodoacetic acid in 1 mol/l sodium hydroxide

was added to the denatured protein solution described above, and the pH adjusted to 8.5 by the addition of 1 mol/l sodium hydroxide. This solution was left for one hour in the dark at 37°C. Prior to applying this sample to the gel filtration column, 3 μ l β mercapto-ethanol were added.

Dissociation of Apoferritin in 67% Acetic Acid

Hofmann and Harrison (1962) dissociated apoferritin in 67% acetic acid and analysed the products by ultracentrifugation in the same solvent, while Crichton and Bryce (1973) dissociated apoferritin in 67% acetic acid, and in some cases acidic buffers, and separated the products by gel filtration in universal buffers in the pH range 1.5 - 5.0. In the work described in this chapter, the sample was dissociated in 67% acetic acid and the products separated by gel filtration in a buffer at pH 1.6.

Initial experiments showed that the preliminary treatment in 67% acetic acid was not essential and that similar elution profiles were obtained when the protein was dissociated in buffer at pH 1.6. However, the original object of these gel filtration experiments was to attempt to separate the components resolved on acetic acid urea electrophoresis, and when these experiments

were carried out the pretreatment for acetic acid urea electrophoresis was 67% acetic acid (Section 5.3).

For dissociation, apoferritin samples (1 mg/0.25 ml) were dissolved in 67% (v/v) acetic acid and left at 4°C for at least one hour. In some experiments, to test the effect of reducing agent on the dissociated products, the dissociating solution was made 1% (v/v) in β mercapto-ethanol.

Separation of the Dissociated Products by Gel Chromatography

Gel Filtration in the Presence of 7 mol/l Guanidine Hydrochloride

The Sephadex G-100 was suspended in 7 mol/l guanidine hydrochloride, and deaerated for several hours before being packed in a Wright's chromatographic column (90 cm \times 1 cm²). The filtration was carried out by upward flow of the eluting solvent (7 mol/l guanidine hydrochloride) which was maintained at a rate of 1.5 ml/h by either the height of the solvent reservoir, or by a peristaltic pump. The sample (0.5 ml) was applied to the column by injecting the material into the bottom of the column by way of a 3-way tap. The eluate was monitored at 280 nm on an LKB ultraviolet absorptionmeter. and fractions (1.8 ml) were collected on an LKB ultrorac fraction collector.

The eluted products were recovered by collecting those fractions which absorbed at 280 nm, and dialysing them against several changes of distilled water at room temperature. Upon removal of the guanidine hydrochloride, the polypeptides precipitated, and these precipitates were lyophilised and stored at -40°C .

Gel Filtration of the Acid Dissociated Products

The Sephadex G-100 column (Wright's chromatography column, $90\text{ cm} \times 1\text{ cm}^2$) was equilibrated in 0.038 M glycine, 0.062 M HCl buffer pH 1.6 containing 0.1 mol/l NaCl. The flow rate of the eluting buffer was 1.5 ml/h , regulated by either the height of the solvent reservoir or by a peristaltic pump. The sample, separated by upward elution, was applied to the column as described above, in a volume of 0.5 ml , and the eluate monitored at 280 nm on an ultraviolet absorptionmeter. Fractions (1.8 ml) were collected on an LKB ultrorac fraction collector and stored at 4°C until analysed by acetic acid urea electrophoresis (Chapter 6).

4.3 Results

Gel Filtration in 7 mol/l Guanidine Hydrochloride

All the ferritin preparations used in this and the following section were purified using isolation procedure 1,

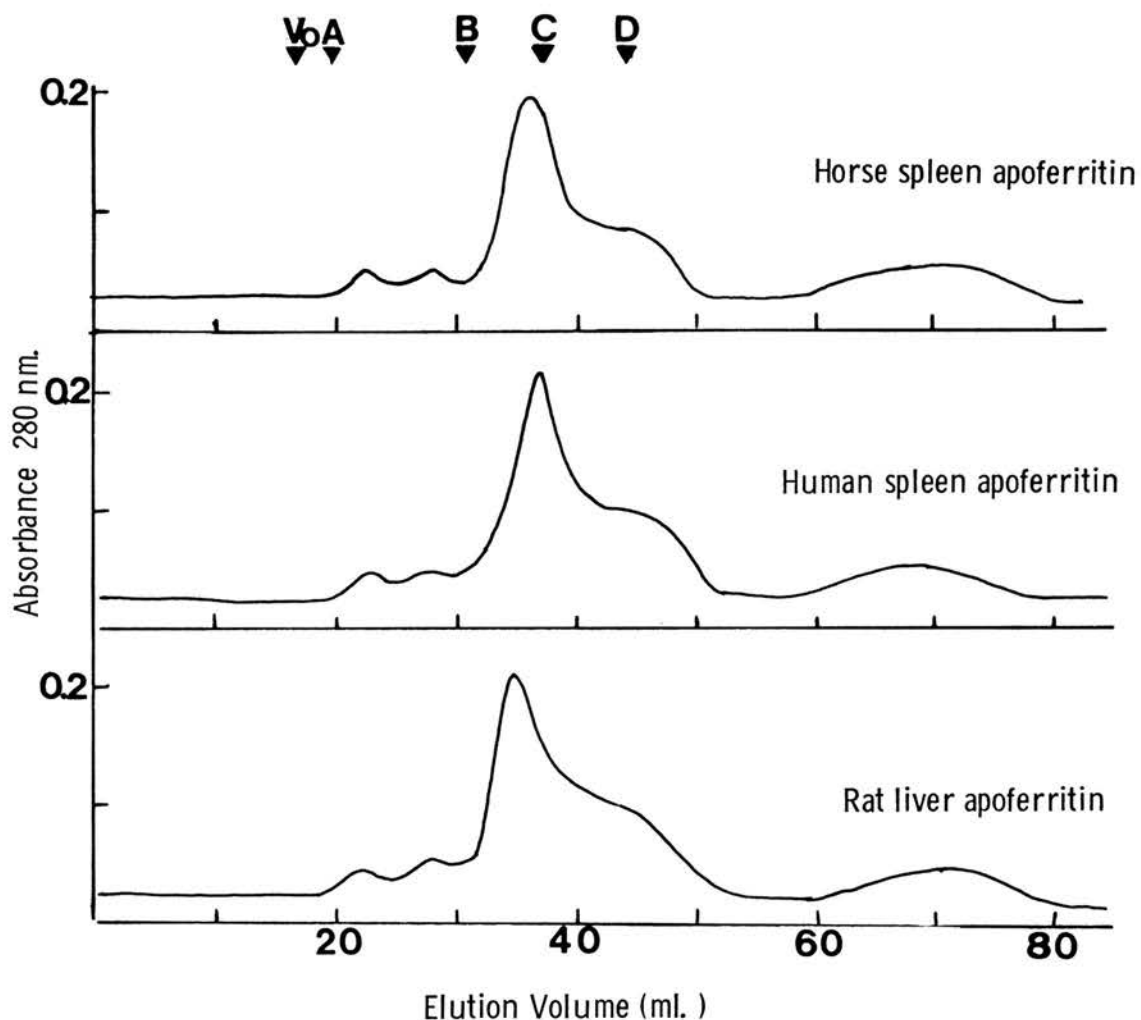


Fig. 6 The elution profiles of rat liver apoferritin, human spleen apoferritin and horse spleen apoferritin, after gel filtration on Sephadex G-100 in the presence of 7Mol/L guanidine hydrochloride. Samples were dissociated in 7Mol/L guanidine hydrochloride, and the sulfhydryl groups were blocked by carboxymethylation, as described in the methods. Also shown is the void volume (Vo), and the volume of the standard proteins A) ovalbumin, B) chymotrypsinogen, C) myoglobin and D) cytochrome c.

Table 2. Elution Data for Gel Filtration on Sephadex
G-100 in the Presence of 7 mol/l Guanidine HCl

| Protein | | V_e (mls) | $V_e - V_o$ (mls) | K_{av} |
|----------------------------|----|-------------|-------------------|--------------|
| Ovalbumin | | 20.3 | 2.3 | 0.03 |
| α -Chymotrypsinogen | | 31.2 | 13.6 | 0.16 |
| Myoglobin | | 38.0 | 20.0 | 0.26 |
| Cytochrome C | | 45.5 | 27.5 | 0.36 |
| Horse Spleen | a) | 36.9 | 18.9 | 0.24 |
| Apo ferritin | b) | ~ 45.0 | ~ 27.0 | ~ 0.351 |
| Human Spleen | a) | 36.9 | 18.9 | 0.24 |
| Apo ferritin | b) | ~ 45.0 | ~ 27.0 | ~ 0.351 |
| Rat Liver | a) | 35.0 | 17.0 | 0.225 |
| Apo ferritin | b) | - | - | - |

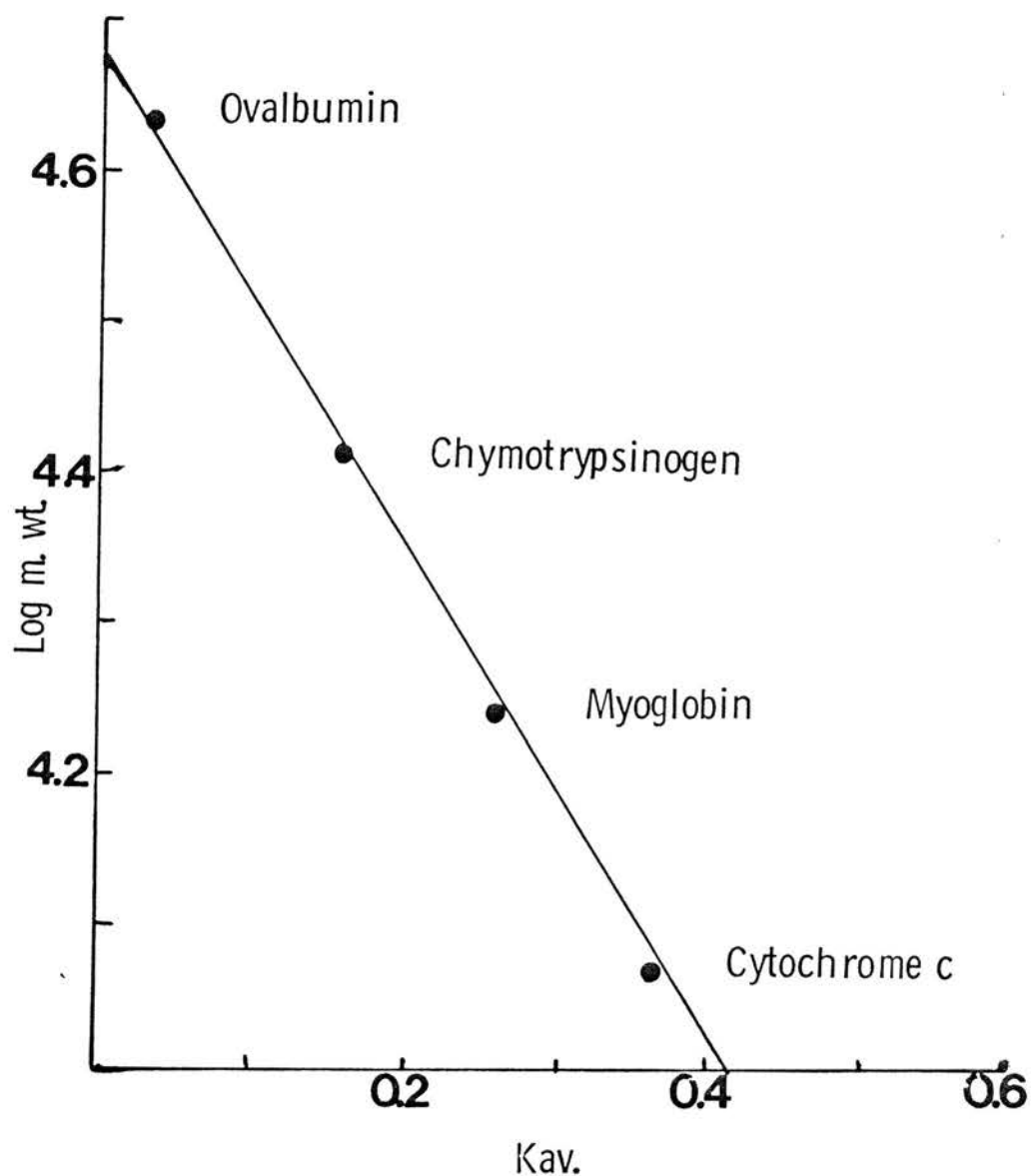


Fig. 7 Standard graph of the Log m. wt. of the proteins versus the distribution coefficients (K_{av}) determined by gel filtration on Sephadex G-100 in the presence of 7 Mol/L guanidine HCl. Column dimensions and flow rate, were as described in the methods.

which involved both ion exchange chromatography and heat treatment stages.

Each gel filtration experiment described here and in the ensuing section, was repeated at least twice on different preparations before the ultimate profile was obtained. The elution profiles of the guanidine hydrochloride dissociated and carboxymethylated horse spleen, human spleen and rat liver apoferritins were almost identical (Fig. 6) with three major 280 nm absorbing peaks, and two smaller peaks close to the void volume. These two smaller peaks were assumed to represent oligomeric species of the dissociated products. The only substantial difference between the elution profiles of the three proteins, was that the rat liver apoferritin main peak was eluted slightly earlier than the two spleen apoferritin main peaks.

The Sephadex G-100 column on which the profiles shown in Fig. 6 were obtained, was calibrated with blue dextran to give the void volume v_o , with tryptophan to give the total column volume v_t , and with the standard proteins; ovalbumin (molecular weight 43,000), α chymotrypsinogen (25,700), myoglobin (17,200) and cytochrome C (11,700). From these data (Table 2) the k_{av} for each standard protein and the apoferritin dissociated material was calculated, where $k_{av} = (v_e - v_o) / (v_t - v_o)$. v_e was the elution volume of the protein under consideration and was

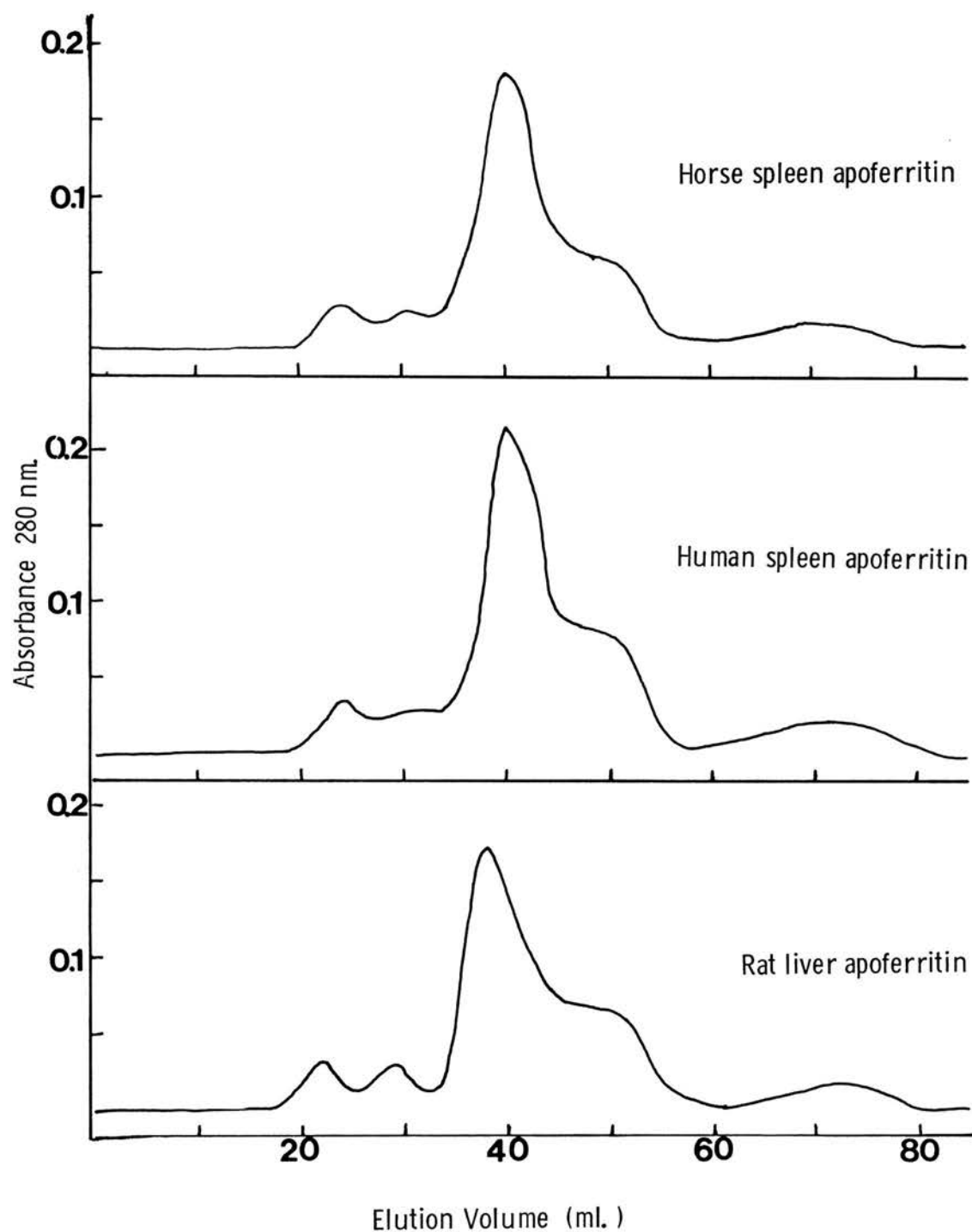


Fig. 8 The elution profiles of rat liver apoferritin, human spleen apoferritin, and horse spleen apoferritin, after gel filtration on Sephadex G-100 in the presence of 7Mol/L guanidine HCl. Samples were dissociated in 7Mol/L guanidine HCl, but the sulfhydryl groups were not blocked by carboxymethylation.

measured as the volume at which the peak height was eluted. The exact elution volume was determined by measuring gravimetrically the volume of each fraction eluted.

The standard graph of the log molecular weight versus k_{av} is shown in Fig. 7. From this linear relationship, the molecular weight of the apoferritin components was determined as being 18,600 for horse and human spleen apoferritin and 19,100 for rat liver apoferritin. The second component in all three apoferritins eluted as a shoulder of the first peak, and so it was not possible to accurately assign a molecular weight to it. However, since its apparent elution volume was close to that of cytochrome C, its molecular weight can be estimated as being approximately in the region 11,000 - 13,000. The third component eluted as a broad peak, to which the assignment of an accurate molecular weight was impossible. The molecular weight, however, relative to the standard proteins, was obviously below 10,000.

The elution profiles of the apoferritin preparations dissociated without carboxymethylation are shown in Fig. 8. These profiles were obtained on a different column from those shown in Fig. 6, and since, for reasons discussed above, these gel filtration experiments were carried out more for preparative than analytical reasons, the columns were not calibrated. Consequently, only

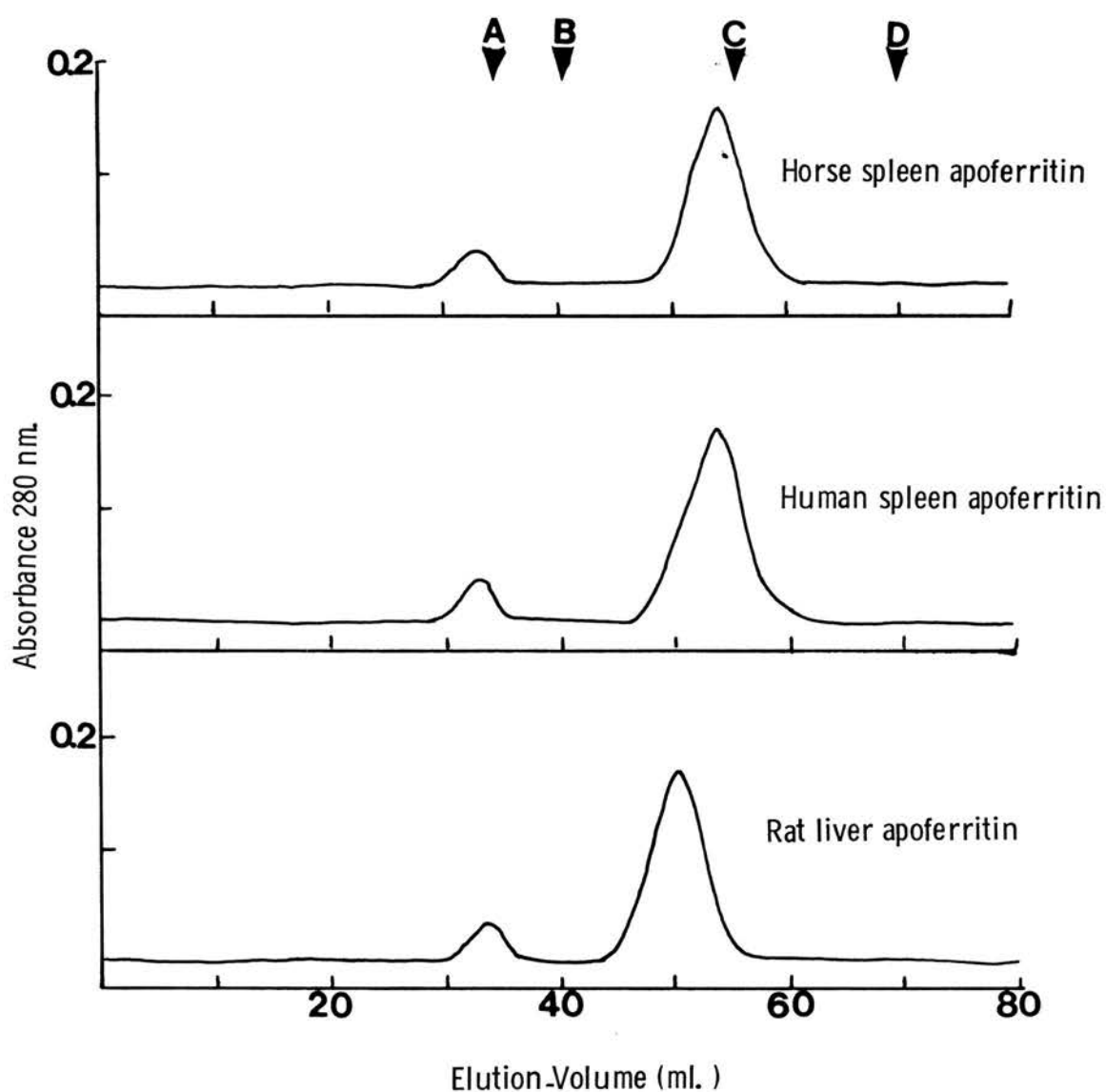


Fig. 9 The elution profiles of rat liver apoferritin, human spleen apoferritin and horse spleen apoferritin, after gel filtration on Sephadex G-100 equilibrated in 0.1 Mol/L glycine HCl buffer pH 1.6. Samples were dissociated in 67% (v/v) acetic acid prior to gel filtration. Also shown is the elution volume of the standards proteins: A) transferrin, B) ovalbumin, C) myoglobin and D) cytochrome c.

general comparisons can be made between these profiles and those shown in Fig. 6. Distinct similarities can be observed between the two sets of profiles with the possible exception that the relative amount of the two smaller peaks eluting near the void volume was greater when the protein was not carboxymethylated. This is not unexpected, since in the absence of carboxymethylation, disulphide bonding would occur, resulting in oligomeric forms of the dissociated polypeptides.

Gel Filtration of Acetic Acid Dissociated Apoferritin on Sephadex G-100 Equilibrated in Acid Buffer

Two absorbance peaks were apparent on the elution profile of horse spleen, human spleen and rat liver acetic acid dissociated apoferritin (Fig. 9). One small peak eluted at the void volume, and was assumed to represent a small amount of undissociated protein, while the other larger absorbance peak represented the dissociated polypeptide. The horse and human spleen dissociated polypeptides had similar elution volumes (54 ml), while the rat liver polypeptide eluted slightly earlier with an elution volume of 50 ml. Calibration of the column with the standard proteins; transferrin (molecular weight 76,000), ovalbumin (43,000), myoglobin (17,300) and cytochrome C (11,200) made it possible to allocate molecular weights of approximately 18 - 19,000 for the two

spleen apoferritin polypeptides, and approximately 18,500 - 19,000 for the rat liver apoferritin polypeptide.

4.4 Conclusion

When the elution profiles obtained from gel filtration in acid are compared with those obtained from gel filtration in 7 mol/l guanidine hydrochloride, the latter gives a more complex dissociation pattern than the acid. No smaller molecular weight components (below 18,000) were eluted from the column equilibrated in acid. The absence of these polypeptides in this system cannot be attributed to the omission of a carboxymethylation step, since when carboxymethylation is omitted from the guanidine hydrochloride system, the smaller polypeptides are still observed. This fundamental difference then would appear to be a property of the dissociation method. Apparently guanidine hydrochloride is a more powerful dissociating agent than acetic acid.

A similarity in the profiles obtained for both systems is that the rat liver apoferritin polypeptide had a slightly higher molecular weight than the horse spleen and human spleen polypeptides, regardless of whether the method of dissociation involved treatment in acid or in 7 mol/l guanidine hydrochloride.

CHAPTER V

ELECTROPHORETIC ANALYSIS OF THE PRODUCTS OF DISSOCIATION OF APOFERRITIN

5.1 Introduction

Two electrophoretic techniques have been developed for use in the analysis of the dissociated constituent polypeptide chains of proteins: polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) (Weber and Osborn, 1969), and acetic acid urea polyacrylamide gel electrophoresis (Panyim and Chalkley, 1969). Further improvement of results can be obtained by subjecting the products separated by either of these methods, to electrophoresis in a second dimension on a gradient polyacrylamide gel.

This chapter describes the results obtained when these techniques were employed to study the subunit structure of apoferritin prepared from horse spleen, human spleen and rat liver ferritins. It also includes studies of the possible effects of certain stages in the preparative techniques, and on the effects of reducing agents and other technical variables on the results of the electrophoretic analyses.

SDS Electrophoresis

The dissociation of proteins by SDS occurs through extensive binding of the hydrophobic region of the negatively charged SDS molecules to the corresponding areas in the polypeptide chains of the protein causing them to

unfold and dissociate. At SDS concentrations above 0.02%, 1.4 g SDS bind per gram of protein, or alternatively, the number of SDS molecules bound to a polypeptide is approximately half the number of amino acid residues in the polypeptide chain (Reynolds and Tanford, 1970a; 1970b; Pitt-Rivers and Impiobato, 1968). This high level of binding and the constant binding ratio in general "swamps out" the negative charge contribution of most proteins, and an approximately constant net negative charge per unit mass is obtained. The properties of the protein-SDS complex as studied by hydrodynamic and optical techniques are consistent with it behaving as a rodlike particle in which the particle length varies with the molecular weight of the polypeptide.

In polyacrylamide gel electrophoresis, separation of proteins depends on two factors, their charge and their mass. (For reviews of PAGE, see Allen and Maurer, 1974 and Chrambach *et al.*, 1976). When proteins are dissociated in SDS, as described above the charge is constant, which permits the separation of SDS dissociated proteins on polyacrylamide gels containing SDS on a molecular weight basis (Shapiro *et al.*, 1967; Weber and Osborn, 1969).

Low molecular weight polypeptides (<10,000) are generally poorly resolved on SDS electrophoresis, and may even migrate with the marker dye (Dunker and Rueckert,

1969; Williams and Gratzer, 1971). In such cases the shape and intrinsic charge of small polypeptides may be relatively more important in determining their mobility in SDS gels than with larger proteins (Swank and Munkres, 1971). Assuming that all proteins bind 1.4 mg SDS per mg protein, it follows that the intrinsic net charge is more important the smaller the peptide, because they bind relatively less SDS on a molar basis. Swank and Munkres (1971) found that improved separation of peptides with molecular weight between 1,200 to 10,000 could be obtained with increased acrylamide concentration and cross-linkage, and the inclusion of 8 mol/l urea to decrease gel porosity.

Acetic Acid Urea Electrophoresis

This electrophoretic technique was originally designed for use in the study of histones (Panyim and Chalkley, 1969), and was based on the cationic electrophoretic system described by Reisfeld *et al.*, (1962), for the separation of basic proteins. The first reported use of acetic acid urea electrophoresis to study the subunit structure of apoferritin (as described in the introduction, Section 1.6) was by Adelman *et al.* (1975).

Gradient and Two-dimensional Electrophoresis

Electrophoresis in polyacrylamide gels containing a

continuous gradient of gel concentration combines electrophoresis and molecular sieving to give high resolution separations. The concentration gradient is formed so that the sample molecules enter a region of the gel of low polyacrylamide concentration and large pore size, and then move into a gel of increasing concentration and decreasing pore size. Molecules of different sizes are sieved out in different positions in the gel, where they are trapped by the smallness of the pore. As the zones move to their final positions, they are automatically sharpened by the gradient (Margolis and Kendrick, 1967).

As a result of this sharpening effect, gradient polyacrylamide gels can be used to advantage in two-dimensional electrophoresis (O'Farrell, 1975), where the protein migrating from the first-dimension gel is sharpened as it passes into the second-dimension polyacrylamide gradient gel. Normally for maximum resolution of proteins the basis for separation in the first dimension should differ from that in the second dimension (O'Farrell, 1975) e.g. acetic acid urea electrophoresis in the first dimension would separate proteins or polypeptides on the basis of their charge to mass ratio, while subsequent electrophoresis in the second dimension on a polyacrylamide gradient gel would separate on a molecular weight basis. However, it is obvious that separation in the two dimensions on the same criterion is

still valuable, e.g. proteins separated on a molecular weight basis, which migrate closely together on a first-dimension SDS gel, may be more clearly resolved by subjecting them to a further electrophoresis in the second dimension on a gradient polyacrylamide gel.

5.2 Methods

The two electrophoretic systems were employed in three different ways:

- (i) PAGE in rods
- (ii) PAGE in slabs of constant polyacrylamide concentration
- (iii) PAGE in slabs in which the polyacrylamide concentration ranged either from 5 to 25% (for SDS) or 7.5% to 25% (for acetic acid urea).

PAGE in the Presence of SDS (Weber and Osborn, 1969)

Preparation of rod gels

Reagents required:

- A. 0.2 mol/l sodium phosphate buffer pH 7.0 containing 0.2% SDS
- B. 22.2 g acrylamide and 0.6 g N,N' methylene-bisacrylamide dissolved in 100 ml distilled water

C. Ammonium persulphate (15 mg/ml)

The gels were cast in 10 cm long glass tubes with an internal diameter of 6 mm. Before use they were soaked in cleaning solution, rinsed and oven dried. For a typical run of 12 gels, 15 ml of reagent (A) was mixed with 13.5 ml of reagent (B) and, after deaeration, 1.5 ml freshly prepared ammonium persulphate solution and 0.045 ml N,N,N',N' tetramethylenediamine (Temed) were added. After mixing, each tube was filled with about 2 ml of the solution. Before hardening, a few drops of distilled water were layered on top of the gel solution.

Preparation of Slab Gels

The procedures described here are for the preparation of batches of homogeneous concentration gels and for concentration gradient gels. The apparatus required for preparing these gels included:

- (i) gel forming tower of the type described by Margolis and Kendrich (1968)
- (ii) gel mould assemblies, consisting of two glass plates ($80 \times 80 \times 1$ mm) held together by waterproof adhesive tape, but spaced 4 mm apart by two $80 \times 4 \times 2.7$ mm glass strips at the outer edges of the plates. These items were obtained

from Universal Scientific Ltd.

(iii) gradient mixer - Pharmacia Gradient Mixer GM-1.

Preparation of Homogeneous Concentration SDS Gels

Reagents required:

- A. 30 g acrylamide and 0.75 g N,N' methylenebis-acrylamide in 300 ml 0.1 mol/l sodium phosphate pH 7.0 containing 0.1% SDS.
- B. 150 ml 5% (v/v) ethanol
- C. 200 ml 25% (w/v) sucrose at 4°C.

The gel mould assemblies were packed into the gel forming tower, and the 5% ethanol solution was admitted to the tower such that the liquid surface was about 1 cm below the bottom edges of the gel cassettes. The ethanol in this solution served several purposes: it helped to free air bubbles from the gel cassettes, it formed a stable interface with the underlying acrylamide, and it gave the gel assemblies a final de-grease.

To prepare the gel solution, 2 ml of a freshly prepared ammonium persulphate solution (100 mg/ml) and 0.15 ml Temed were added to solution (A) and this polymerising solution was then deaerated. This was slowly introduced into the gel moulds by upward displacement of

the 5% (v/v) ethanol, and adjusted to its correct height in the mould by the level of the dense sucrose solution (C).

Preparation of Concentration Gradient SDS Gels

Reagents required:

G₁. 7.5 g acrylamide and 0.189 g N,N' methylenebisacrylamide in 150 ml 0.1 mol/l sodium phosphate pH 7.0 containing 0.1% SDS.

G₂. 37.5 g acrylamide and 0.937 g N,N' methylenebisacrylamide in 150 ml 0.1 mol/l sodium phosphate pH 7.0 containing 0.1% SDS.

B. 150 ml 5% (v/v) ethanol

C. 200 ml 25% (w/v) sucrose at 4°C.

The 5% ethanol solution was admitted to the tower as described above. After deaeration, solutions G₁ and G₂ were then added to the respective reservoirs of the gradient mixer. 1 ml of a freshly prepared ammonium persulphate solution (120 mg/ml) and 0.1 ml Temed were added to the solutions in each reservoir and the gradient allowed to form. The freshly mixed gradient was then admitted into the tower by upward displacement of the 5% (v/v) ethanol, and adjusted to its correct height in

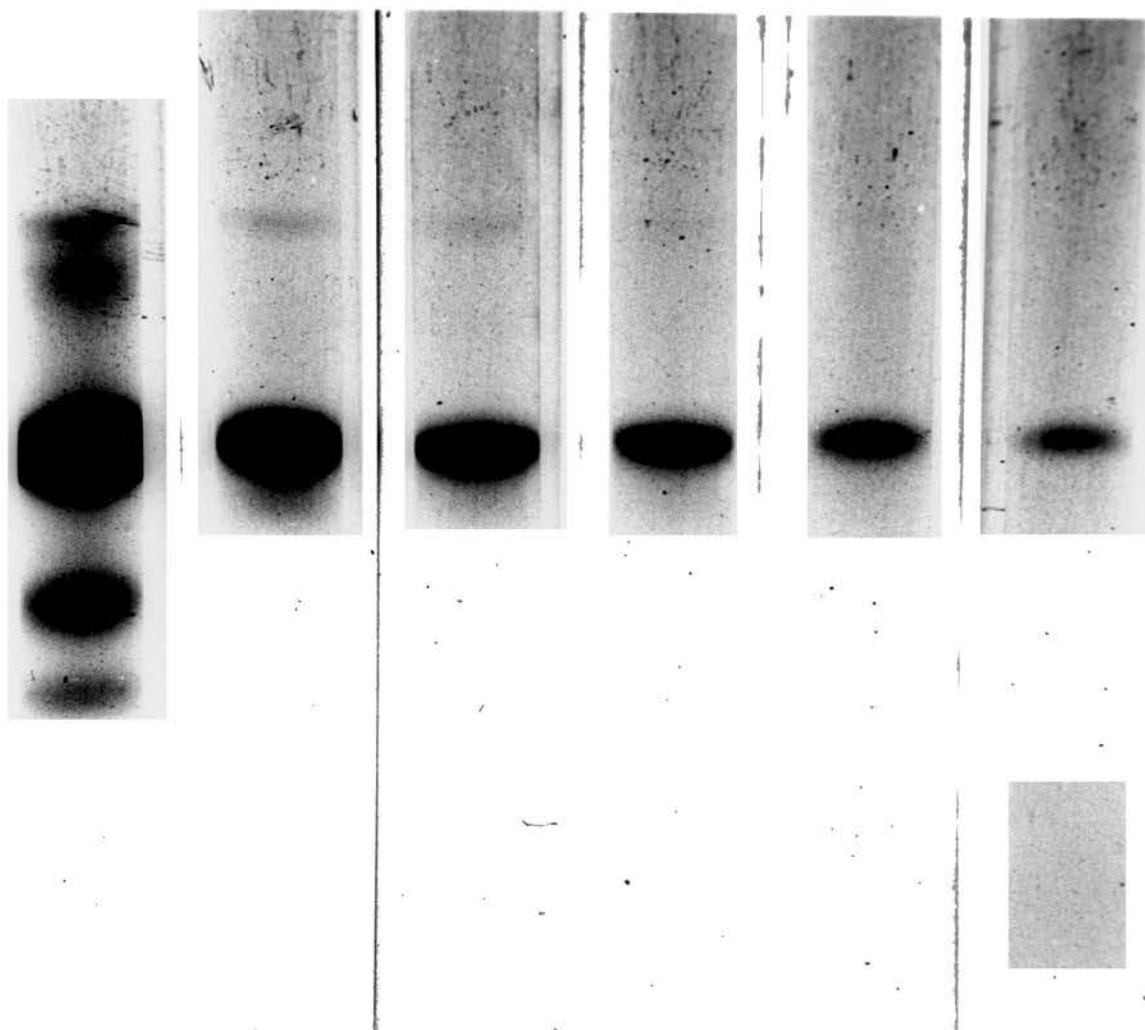


Fig. 10 SDS polyacrylamide gels after electrophoresis of horse spleen apoferritin. The quantity of protein applied to each gel was, from L to R : 125 ug, 62 ug, 31 ug, 15 ug, 7 ug and 4 ug.

the moulds by the level of the dense chase solution (C).

SDS gradient gels have been used successfully by Maurer (1971) Voyles and Moskowitz (1974) and Anderson *et al.* (1974).

Preparation of the Sample

Routinely the protein samples (1 mg) were incubated at 70°C for 30 minutes in 1 ml of 0.1 mol/l sodium phosphate pH 7.0 containing 1% SDS and 1% β mercaptoethanol. However, in those experiments in which the effect of β mercaptoethanol and dithiothreitol on the banding pattern was studied, the protein sample was dissolved in 0.1 mol/l sodium phosphate buffer pH 7.0 containing 1% SDS, and the appropriate amounts of reducing agent (Section 5.3) were added, allowances being made to ensure that sample volumes were uniform.

Before application to the gel, 3 μ l of tracking dye (0.05% w/v bromophenol blue) and one drop glycerol were added to the sample. 15 to 20 μ l of this sample, corresponding to about 15 μ g protein were applied to the gel.

While it is important not to put too much protein on SDS gels, since overloading may cause artefacts, it is equally true that significant observations may be missed if insufficient material is put on the gel. This is illustrated in Fig. 10, which shows the electrophoretic

pattern obtained when quantities ranging from 5 to 125 μg of a horse spleen apoferritin preparation made by R. R. Crichton were analysed by SDS electrophoresis. Crichton (personal communication) believed that this material did not give the multiple bands described in other laboratories (Niitsu *et al.*, 1973; Ishitani *et al.* 1975; Linder *et al.*, 1974; Adelman *et al.*, 1975), but the additional bands apparently in the lower molecular weight range are easily seen in all gels containing 15 μg protein or more. For this reason it was felt important to standardise the amount of protein applied to each gel at approximately 15 μg .

Running Conditions

The electrode buffer was 0.1 mol/l sodium phosphate pH 7.0 containing 0.1% SDS. Rod and homogeneous concentration slab gels were pre-run at 3 mA/gel for one hour and electrophoresis was performed at 8 mA/gel for 6 hours for rods, and 25 mA for 6 hours for slabs. The gradient gels were pre-run at 25 mA for 2-4 hours and electrophoresis performed at 50 mA for at least 12 hours.

Staining and Destaining

Gels were stained for protein in a solution containing 1.25 g Coomassie Brilliant Blue R in a mixture of 454 ml

50% (v/v) methanol and 46 ml glacial acetic acid. Staining was at room temperature for 2-10 hours. Destaining solution contained 75 ml glacial acetic acid, 50 ml methanol and 875 ml distilled water. Rod gels were destained at 45°C by repeated changes of destainer. The slab gels were destained electrophoretically in a Uniscil electrophoretic destaining apparatus at room temperature.

Scanning of Gels

Gels stained for protein either by Coomassie Brilliant Blue or Napthalene Black were scanned at 580 nm (Unicam Sp 500 with Gilford Scanner attachment). The scan speed was 2 cm/min, while the chart speed was 4 cm/min.

Acetic Acid Urea PAGE (Panyim and Chalkley, 1969)

Preparation of Rod Gels

Reagents required:

- A. 60% (w/v) acrylamide and 0.4% (w/v) N,N' methylene-bisacrylamide in distilled water
- B. 43.2% (v/v) glacial acetic acid and 4% (v/v) Temed in distilled water
- C. 8 mol/l urea

D. ammonium persulphate (50 mg/ml).

Solutions (A) and (B) were stored at 0° and warmed to room temperature before use. To prepare the gel solution, the reagents were mixed in the ratios 1 part (B) to 2 parts (A) to 5 parts (C). 1 ml of a fresh ammonium persulphate solution was then added, and the gel solution deaerated.

The gels were cast in glass tubes (10 cm × 6 mm) which were soaked in cleaning solution, rinsed and oven dried before use.

Preparation of Concentration Gradient Acetic Acid Urea Gels

The polyacrylamide gradient selected was 7.5-25%, since 5-5% gradients gelled unevenly across their width.

Reagents required:

G₁. 30% (w/v) acrylamide and 0.8% (w/v) N,N' methylene-bisacrylamide in distilled water

G₂. 100% (w/v) acrylamide and 2.64% (w/v) N,N' methylene-bisacrylamide in distilled water

B. 43.2% (v/v) glacial acetic acid and 4% (v/v) Temed

C. 8 mol/l urea

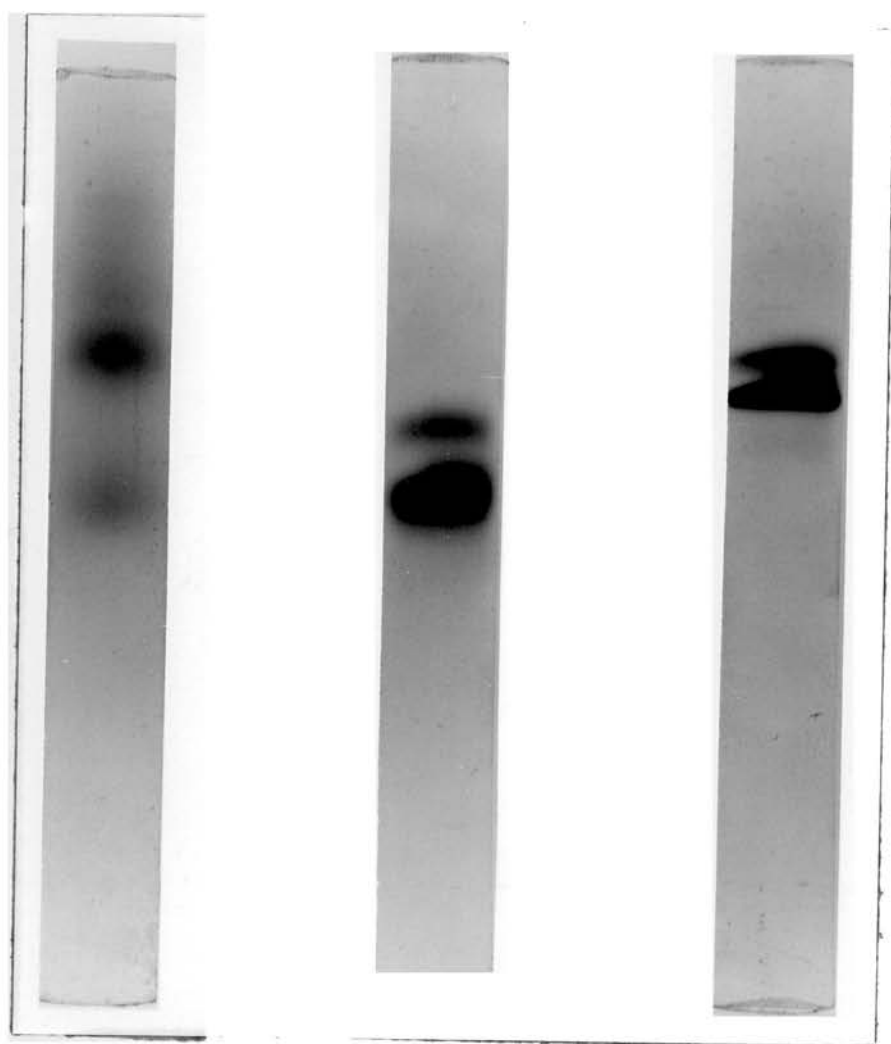


Fig. 11 Acetic acid urea gels after electrophoresis of human spleen apoferritin. The gels contained from L to R, 0 Mol/L urea, 2.5 Mol/L urea and 5 Mol/L urea.

- D. 5% (v/v) ethanol
- E. 50% (w/v) sucrose
- F. ammonium persulphate (100 mg/ml)

To prepare 15 gradient gels, one reservoir of the gradient mixer contained; 40 ml G_1 , 18.5 ml (B) and 92 ml (C), while the other reservoir contained 40 ml G_2 , 18.5 ml (B) and 92 ml (C).

Polymerisation was initiated by the addition of 1 ml of freshly prepared ammonium persulphate (F) to the solutions in each reservoir. The gradient gel was then poured, as described for the SDS gradient gel, by upward displacement of 5% (v/v) ethanol in the gel forming tower, and the height of the gel in the moulds adjusted by altering the level of the dense sucrose chase solution.

In both the rod and gradient acetic acid urea gels, the concentration of urea routinely used within the gel was 5 mol/l. In preliminary experiments the effect of the concentration of urea in the gel on the electrophoretic behaviour of human spleen apoferritin was studied. Gels were used in which the concentration of urea was altered by adjusting the concentration of the stock urea solution. The band patterns obtained in gels containing 0, 2.5 and 5 mol/l urea are illustrated in Fig. 11. With an increase in the amount of urea within the gel, the sharpness of

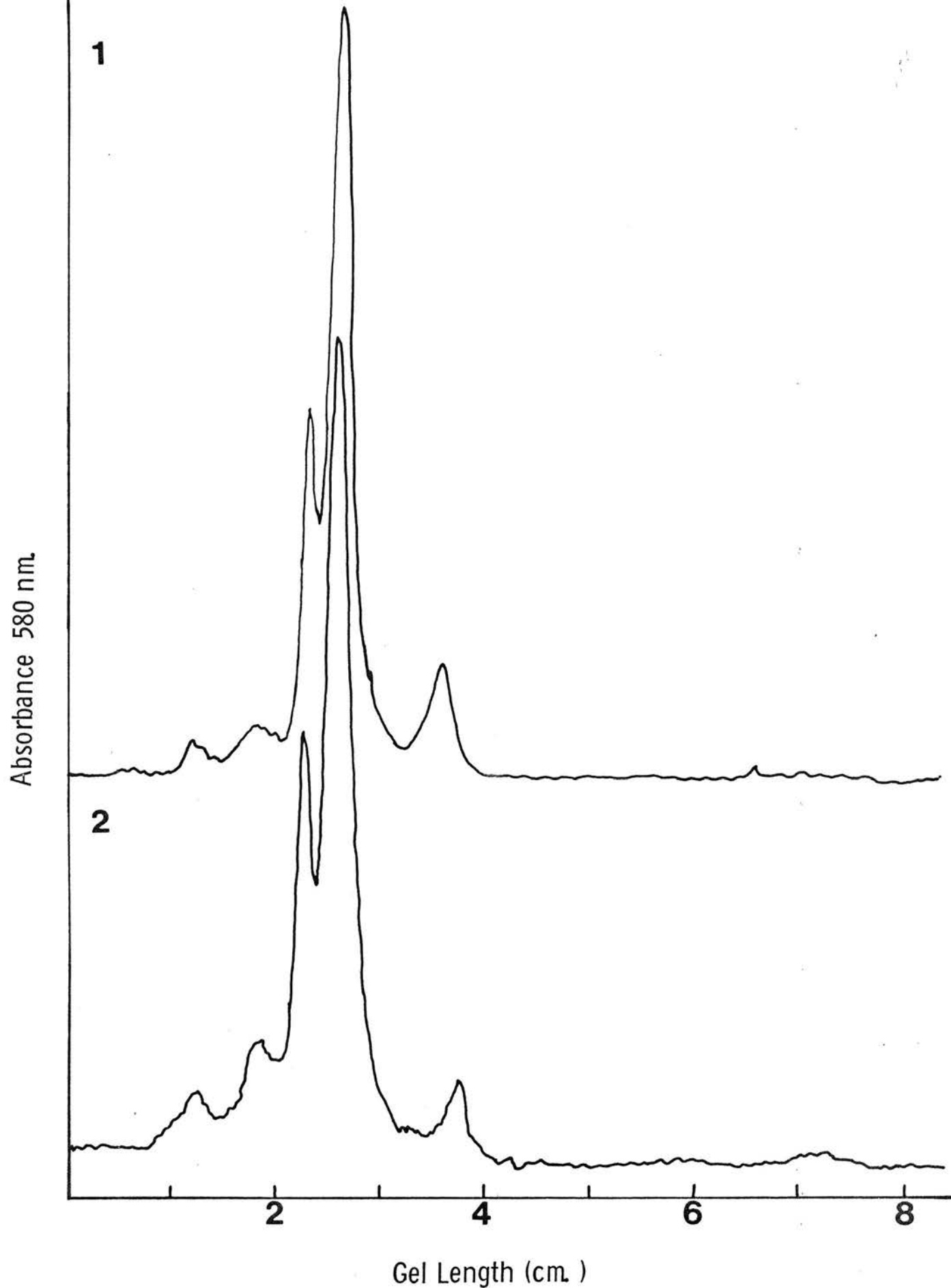


Fig. 12 Densitometric scans of stained acetic acid urea polyacrylamide gels after electrophoresis of horse spleen apoferritin. Before application to the gel, samples were dissolved in:

1) 67%(v/v) acetic acid

2) 0.1 Mol/L sodium phosphate buffer pH 7.

the bands is intensified.

Preparation of the Sample

Sample pre-treatment in 67% (v/v) glacial acetic acid was used, since this was the method of dissociation employed by Harrison and Hofmann (1962) in their ultra-centrifugal work, and seemed the best dissociation method to use in an electrophoretic system run under acidic conditions.

Routinely, the protein was dissolved (1 mg/ml) in 67% (v/v) glacial acetic acid and allowed to stand for one hour at 4°C. Before application to the gel, 10 μ l 25% (w/v) sucrose solution was added to 0.2 ml of the sample solution, and 15 to 20 μ l of this sample (about 15 μ g protein) was applied to the gel.

However, it was found that this sample pre-treatment was not essential to show dissociated components on acetic acid urea gels. Acetic acid urea electrophoresis of horse spleen apoferritin after treatment with either 67% (v/v) acetic acid, or 0.1 mol/l sodium phosphate buffer phosphate buffer pH 7.0 is shown in Fig. 12. It would appear that dissociation of the protein prior to acetic acid urea electrophoresis is not necessary, and that the acid conditions of the electrolyte solution (0.9 mol/l acetic acid, pH 3.4) and within the gel

(pH 3.4) are sufficient to cause dissociation.

Running Conditions

The electrolyte solution was 0.9 mol/l acetic acid. Gels were pre-run overnight at 50 v, while electrophoresis was performed at a total voltage of 100 volts for 4 hours for rod gels and at 100 volts for at least 12 hours for gradient gels. Under these conditions the protein sample migrated towards the cathode.

Staining and Destaining

Gels were stained for a minimum of 5 hours in 0.1% naphthalene black in the destaining solution, which contained methanol, water and glacial acetic acid (5:5:1). Destaining was carried out at 45°C with repeated changes of destainer, while the gradient gels were electrophoretically destained.

Two-dimensional Electrophoresis

Two-dimensional electrophoresis was performed in two ways:

- (a) when both dimension gels contained the same reagents, e.g. SDS rod gels in the first dimension and SDS gradient in the second, and acetic acid

urea rod gels in the first dimension and acetic acid urea gradient in the second.

- (b) when the reagents were different in the two dimensions, e.g. acetic acid urea rod gels in the first dimension and SDS gradient in the second.

In both systems, the first-dimension gel was run in duplicate. One gel was stained, while the other was cut longitudinally, and one half placed on top of the second-dimension gradient gel. If both gels were of the same type, no treatment was required between the two electrophoreses. However, before an acetic acid urea gel was subjected to electrophoresis in the second dimension on an SDS gradient gel, it was dialysed for at least two hours against a solution containing 0.1% SDS in 0.1 mol/l sodium phosphate pH 7.0. This was to allow SDS to bind to the separated first-dimension components (Drysdale *et al.*, 1977).

5.3 Results

SDS Electrophoresis of Apoferritin

Preliminary experiments were carried out on human spleen apoferritin prepared by isolation procedure (1) involving heat treatment and ion exchange chromatography.

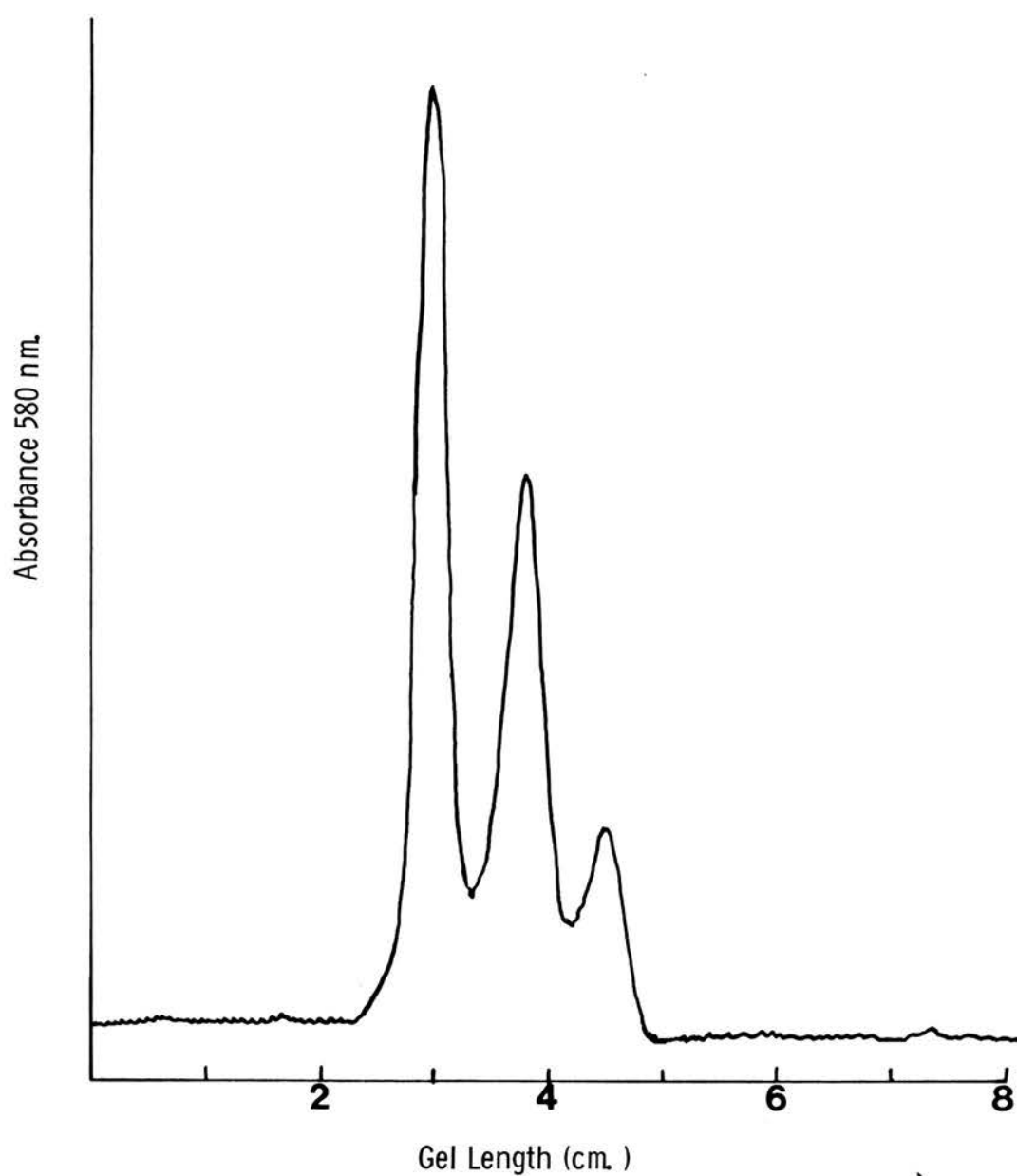


Fig. 13 Densitometric scan of a stained SDS polyacrylamide gel, after electrophoresis of human spleen apoferritin isolated by method 1), which involved heat treatment and cm-cellulose chromatography.

Table 3. The Relative Mobility of Standard Proteins and Human Spleen Apoferritin Components on SDS PAGE, where the Relative Mobility

$$= \frac{\text{distance protein migration}}{\text{length after staining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

| Protein | m.wt. | Relative Mobility |
|--------------------------|--------|-------------------|
| Transferrin | 46,000 | 0.175 (14 gels) |
| Ovalbumin | 43,000 | 0.380 (11 gels) |
| Myoglobin | 17,000 | 0.770 (15 gels) |
| Cytochrome C | 11,700 | 0.950 (5 gels) |
| Human Spleen Apoferritin | | |
| Component 1 | | 0.750 (6 gels) |
| Component 2 | | 0.720 (6 gels) |

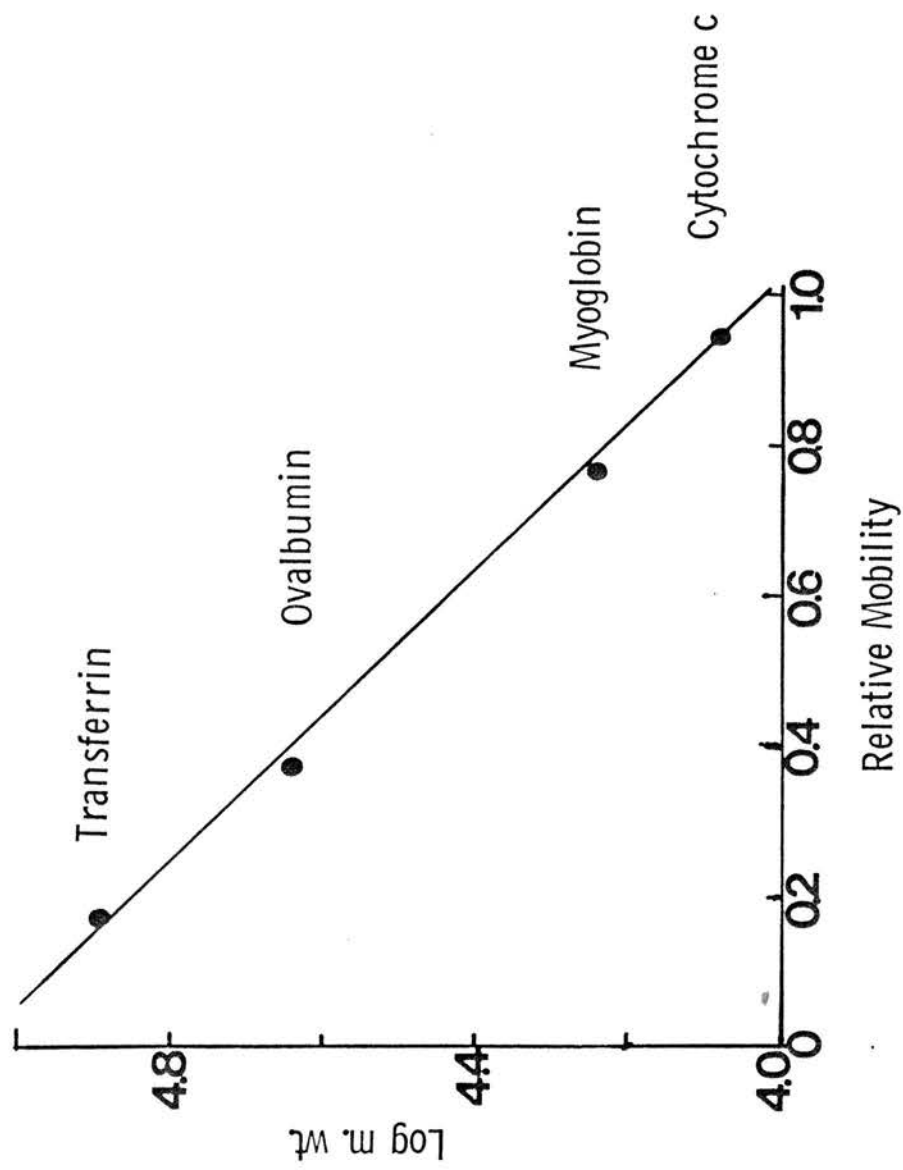


Fig14 Standard graph of Log m. wt. versus relative mobility of proteins on SDS polyacrylamide gel electrophoresis. The running conditions, and the method used to determine the relative mobility are described in the text

When this protein was subjected to SDS electrophoresis, three well-defined protein staining peaks were obvious on scans of the gels (Fig. 13). In order to ascertain the molecular weight of these separated components, the standard proteins transferrin (molecular weight 76,000), ovalbumin (43,000), myoglobin (17,300) and cytochrome C (11,600) were also subjected to SDS electrophoresis and the mobilities of these proteins and the apoferritin components relative to bromophenol blue were obtained (Table 3). From a graph of the log molecular weight versus relative mobility (Fig. 14), the molecular weight of the apoferritin components was estimated at approximately 18,500 and 12,300. The molecular weight of the component corresponding to the third band was below 10,000, and therefore within the limitations of this technique, could not be determined accurately (Dunker and Rueckert, 1969; Williams and Gratzner, 1971), although Niitsu *et al.* (1973), from its migration rate on SDS gels, claimed that it had a molecular weight of approximately 6-7,000.

Effect of Reducing Agent

To examine the effect of reducing agent on the SDS electrophoretic banding pattern of human spleen apoferritin, the protein samples (1 mg) were dissolved in 1 ml of 0.1 mol/l sodium phosphate buffer pH 7.0 containing

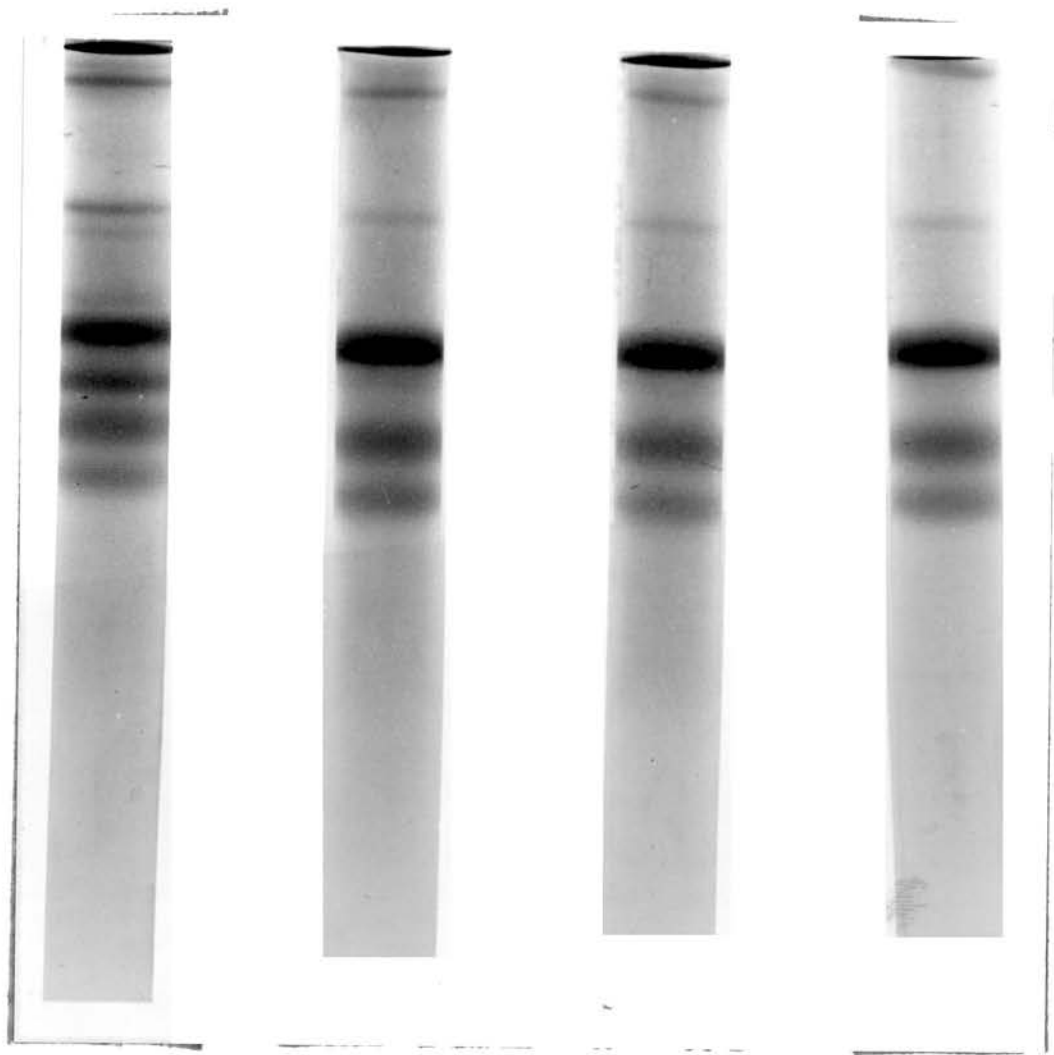


Fig. 15 SDS polyacrylamide gels after electrophoresis of human spleen apoferritin isolated by method 1). Samples were treated with, from L to R, 0, 140, 280 and 560 μ moles 2-mercaptoethanol per ml. dissociating reagent.

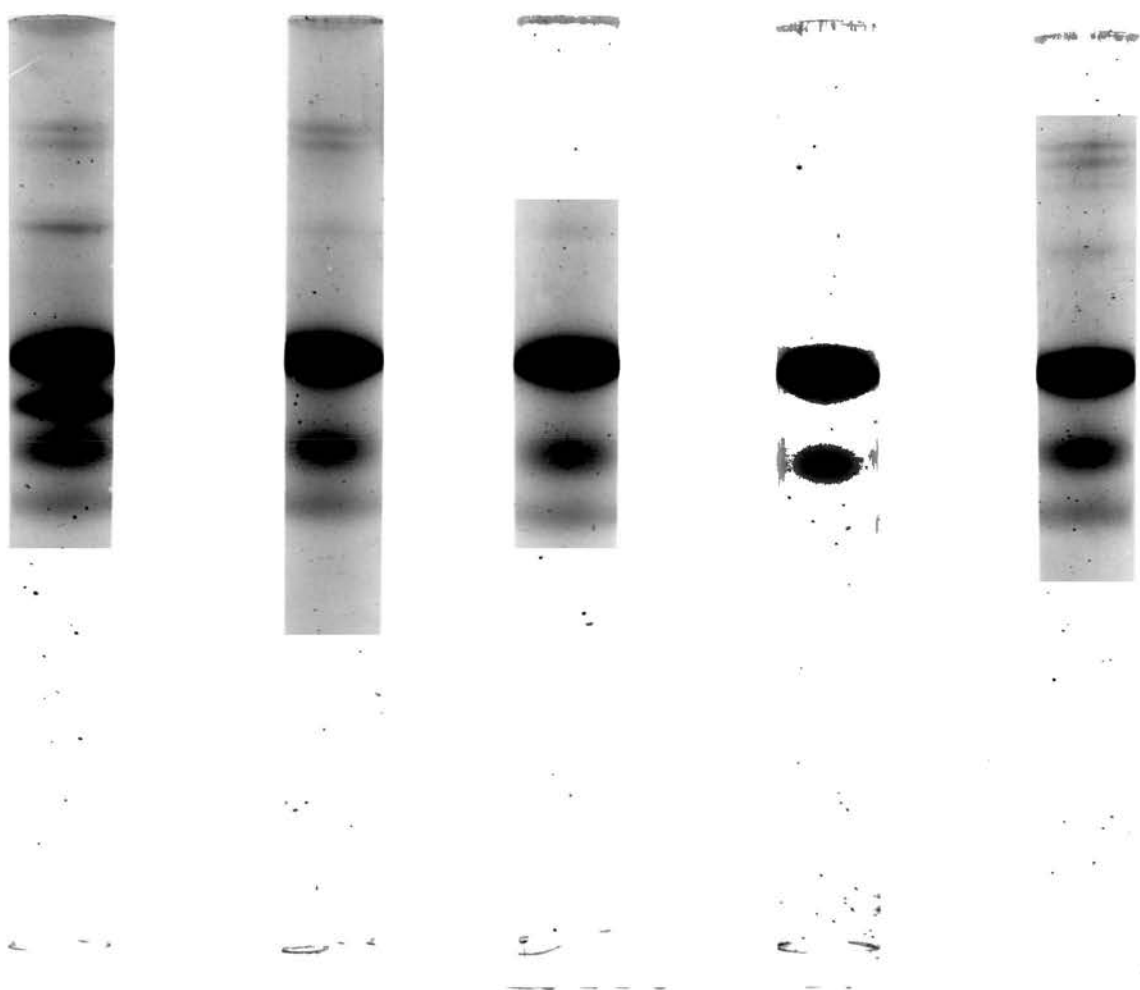


Fig. 16 SDS polyacrylamide gels after electrophoresis of human spleen apoferritin isolated by method 1). Samples were treated with, from L to R, 0, 25, 50, 100 and 250 μ moles dithiothreitol per ml. dissociating reagent.

1% SDS, and divided into 5 aliquots of 0.2 ml. In one experiment 0, 28, 56 and 110 μ moles of β -mercaptoethanol, and in another 0, 5, 10, 20 and 50 μ moles of dithiothreitol were added to these samples.

The effect of β -mercaptoethanol and DTT were similar (Figs. 15 & 16). In the absence of reducing agent four major bands were observed, corresponding to components of approximate molecular weight 18,500; 15,000, 12,300 and "6-7,000". There were also several subsidiary bands above the 18,500 molecular weight component which probably represented oligomeric species (Niitsu *et al.*, 1973). When reducing agent was present in the incubating samples, the 15,000 m.wt species disappeared, while the amount of the subsidiary bands progressively weakened as the concentration of reducing agent was increased. These results confirm that the use of 1% (v/v), 140 μ moles/ml, β -mercaptoethanol in the standard method of sample preparation (Weber and Osborn, 1969) was a sufficient amount to ensure reduction of disulphide bonds, since the banding pattern obtained for samples treated with this standard amount, were similar to those for samples with four times this amount.

Effect of Method of Ferritin Isolation

The experiments described in this section were

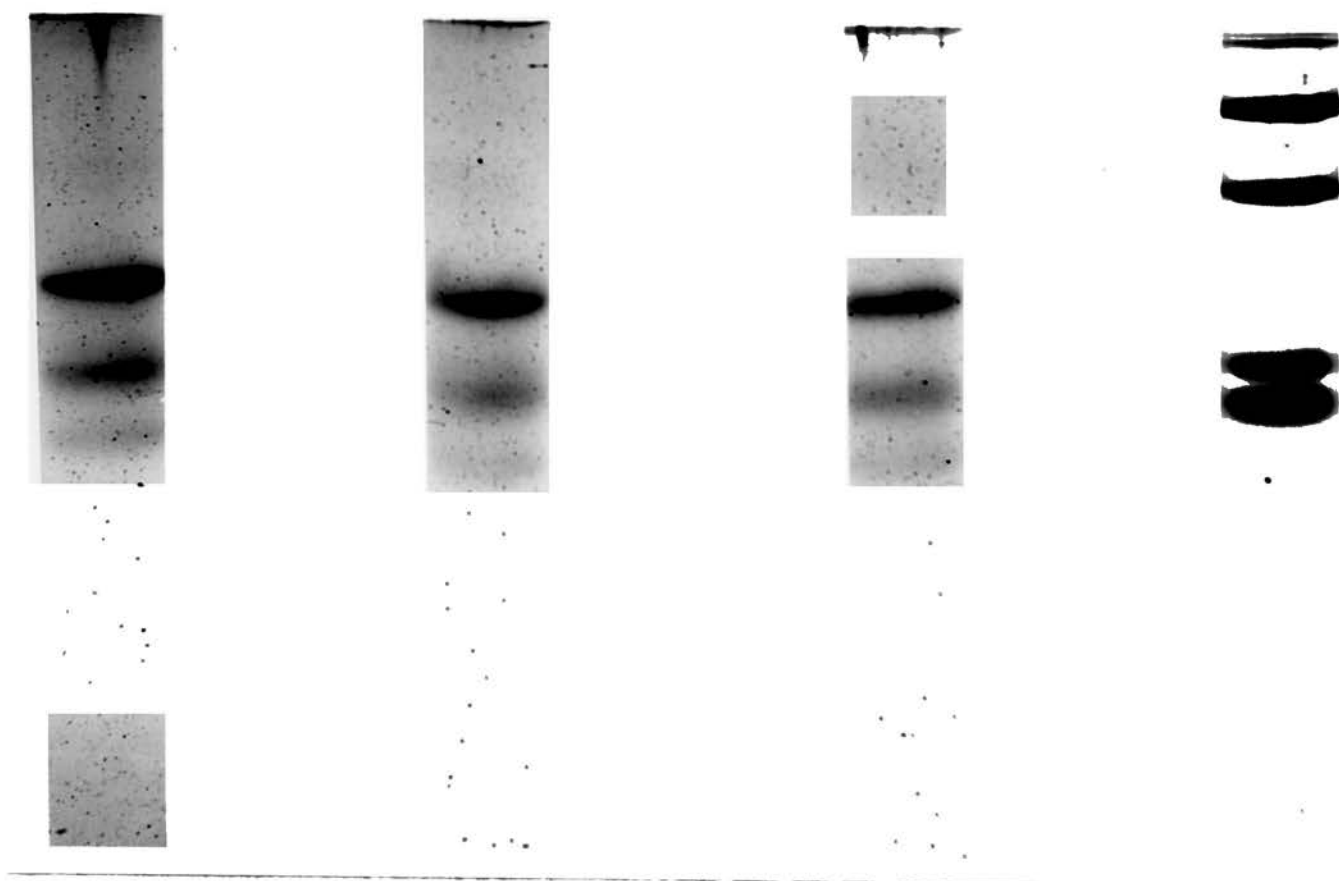


Fig. 17 SDS polyacrylamide gels after electrophoresis of human spleen apoferritin isolated by, from L to R, methods 1), 2) and 3). The fourth gel shows the standard proteins transferrin, ovalbumin, myoglobin and cytochrome c.

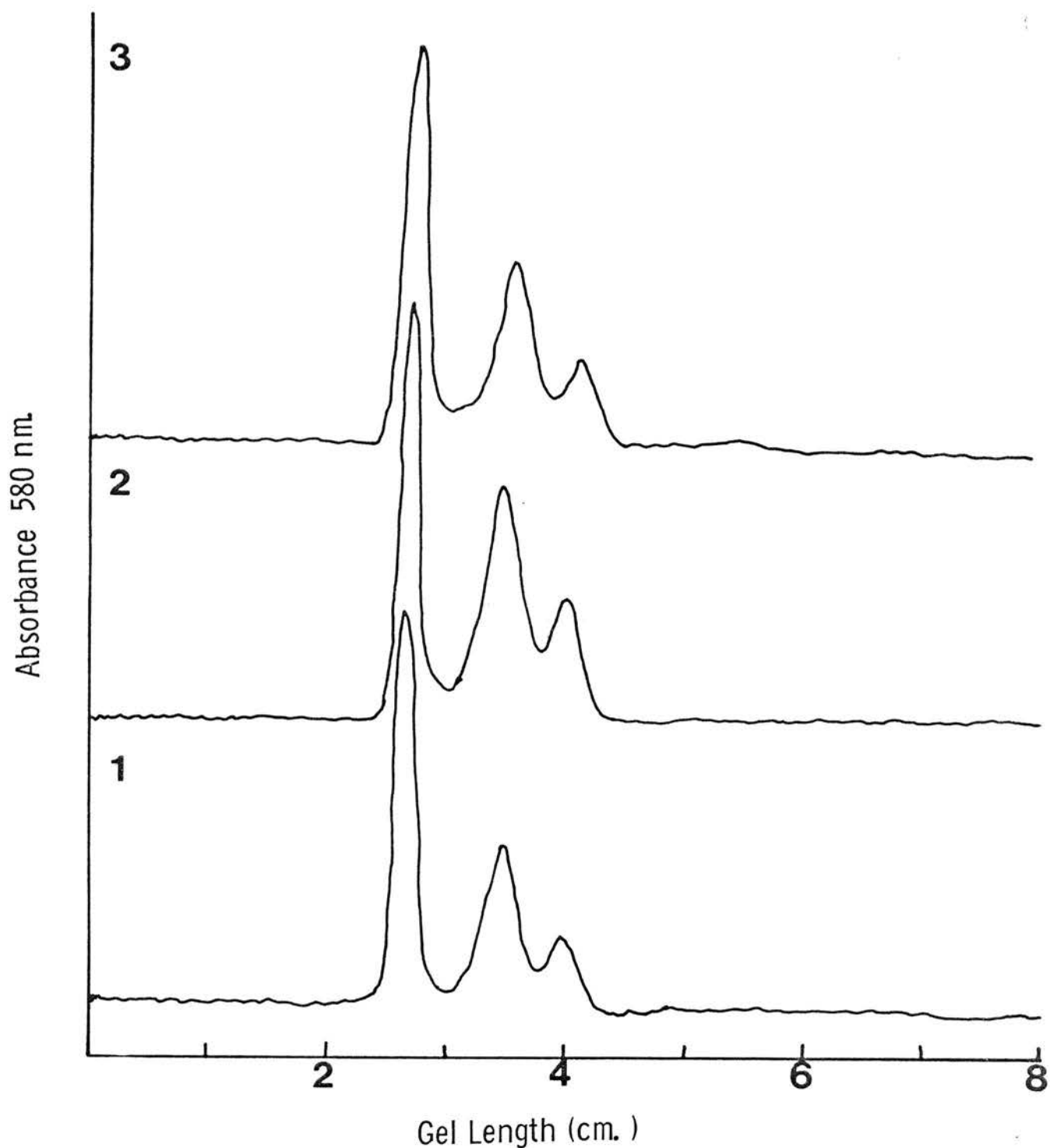


Fig. 18 Densitometric scans of stained SDS polyacrylamide gels, after electrophoresis of human spleen apoferritin isolated by the following procedures:

- 1) Method 1), which involved heat treatment and cm-cellulose chromatography
- 2) Method 2), which involved heat treatment, but omitted cm-cellulose chromatography
- 3) Method 3, which involved cm-cellulose chromatography, but omitted heat treatment.

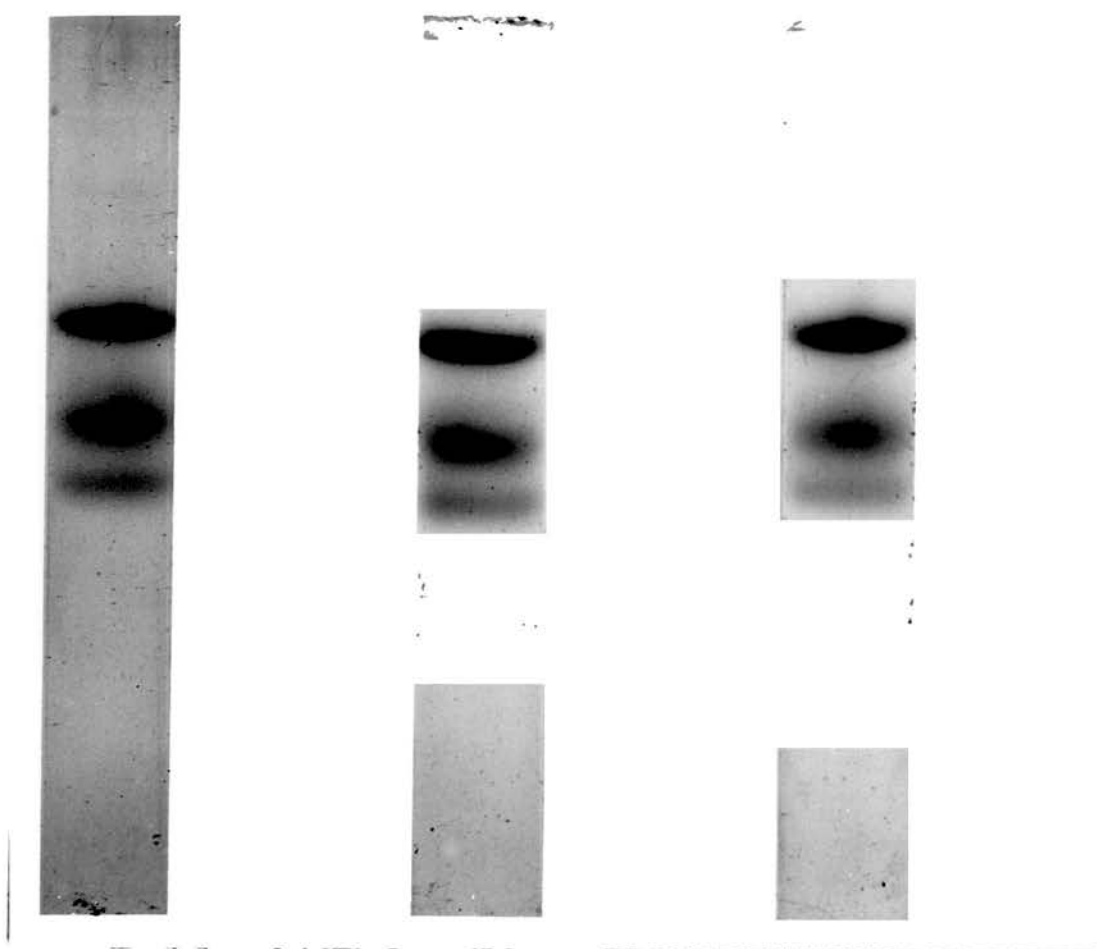


Fig. 19 SDS polyacrylamide gels after electrophoresis of horse spleen apoferritin isolated by, from L to R, methods 1), 2) and 3).

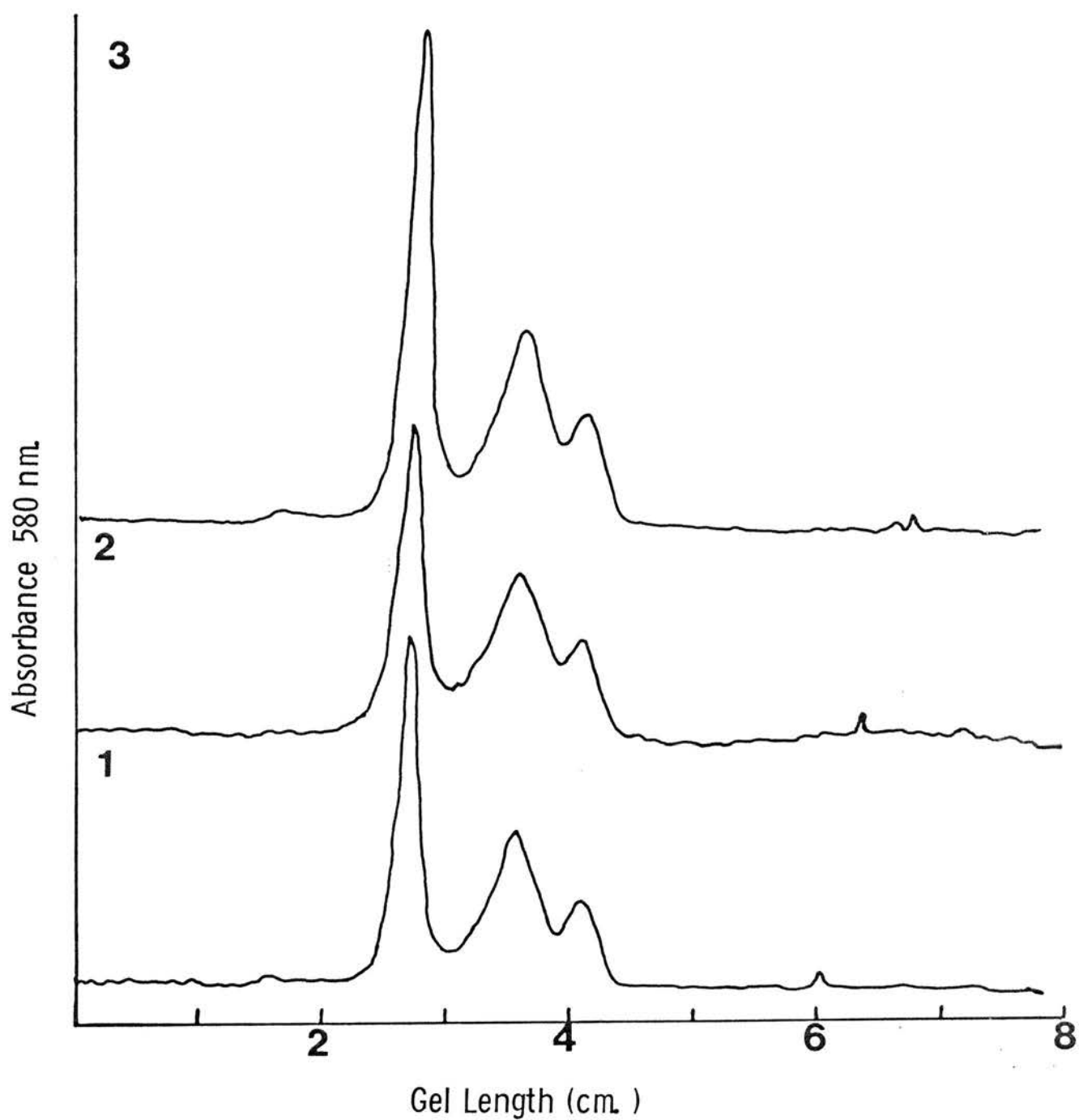


Fig. 20 Densitometric scans of stained SDS polyacrylamide gels , after electrophoresis of horse spleen apoferritin isolated by the following procedures:

- 1) Method 1)
- 2) Method 2)
- 3) Method 3)

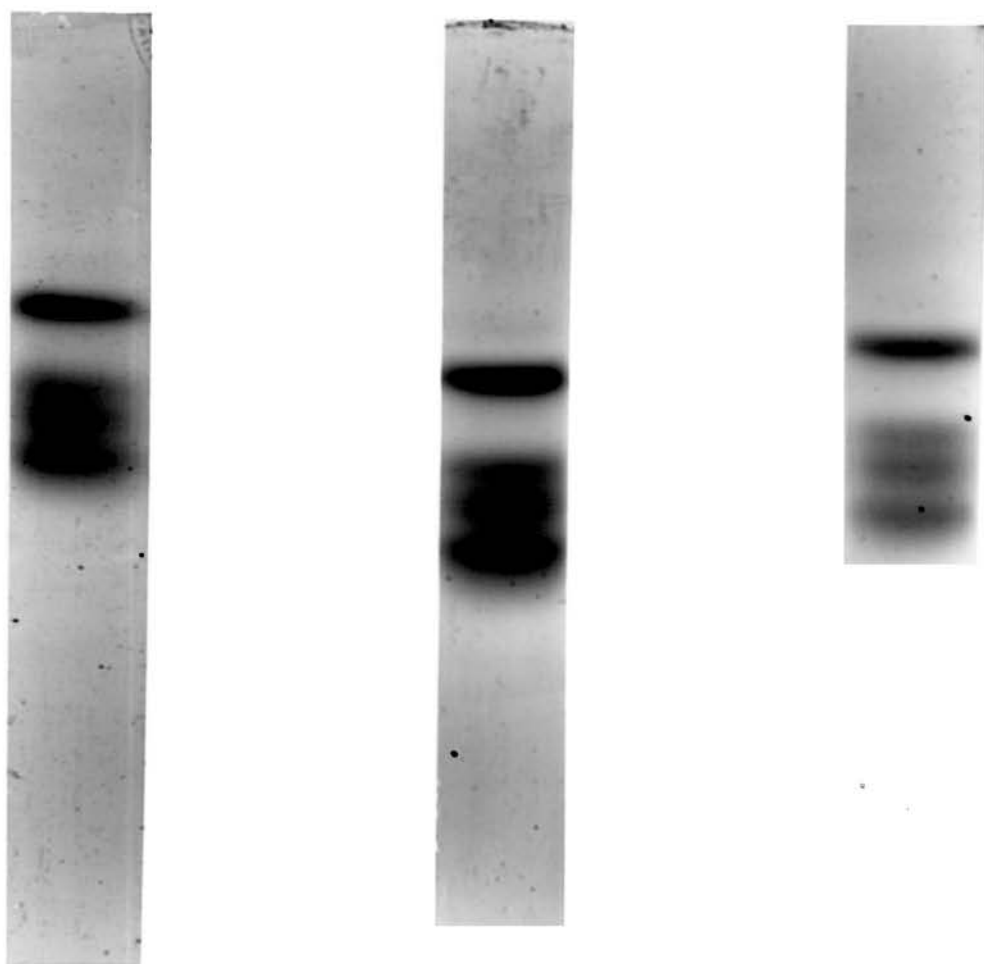


Fig. 21 SDS polyacrylamide gels after electrophoresis of rat liver apoferritin isolated by, from L to R, methods 1), 2) and 3).

designed with two aims in mind:

- (i) to determine whether ferritin prepared from horse spleen and rat liver could, like that prepared from human spleen, be resolved into multiple components by SDS electrophoresis,
- (ii) to determine whether the method used to isolate ferritin would affect the subsequent banding pattern.

The SDS electrophoretic banding pattern of horse spleen, human spleen and rat liver apoferritin isolated by the three methods described in Chapter 3, are shown in Figs. 17-21. Considering the two spleen ferritins first, the horse spleen apoferritin was separated into three components whose migration rate and staining intensity was identical to that of human spleen apoferritin. Moreover, the migration rates and staining intensities of the components of ferritins prepared by the three methods were also identical. The lack of variation in the apparent amount of the smaller components with the method of isolation fails to confirm reports by Crichton (Collett-Cassart and Crichton, 1975) that these are the results of proteolytic cleavage of the main component, occurring during the heat treatment stage of the isolation procedure. Isolation procedure (iii) avoids the use of heat treatment, and yet the ferritins prepared by this

method contain as much of these smaller components as ferritins prepared by methods involving heat treatment.

The rat liver ferritin results are reported separately from the two spleen ferritins, because it behaves differently on SDS electrophoresis. Regardless of the method of isolation four protein staining bands were observed (Fig. 21), even although each sample was treated in the standard manner (with 1% (v/v) β mercaptoethanol) before application to the gel. The molecular weight of these four components was approximately 18,500; 15,000; 12-13,000 and below 10,000. Unlike the two spleen ferritins, in which case the 15,000 molecular weight species disappears on treatment with reducing agent, in rat liver ferritin this component persists when treated with reducing agent. This observation indicates a difference in the disulphide bonding properties between the SDS dissociated components of rat liver apoferritin and the two spleen apoferritins.

Gradient SDS Electrophoresis

By virtue of its band sharpening properties gradient pore should be able to separate more clearly bands which might not be quite as well resolved as homogeneous concentration gels. An SDS gradient pore gel of horse spleen, human spleen and rat liver apoferritin is shown in Fig. 22. The apoferritin samples were all separated into a major

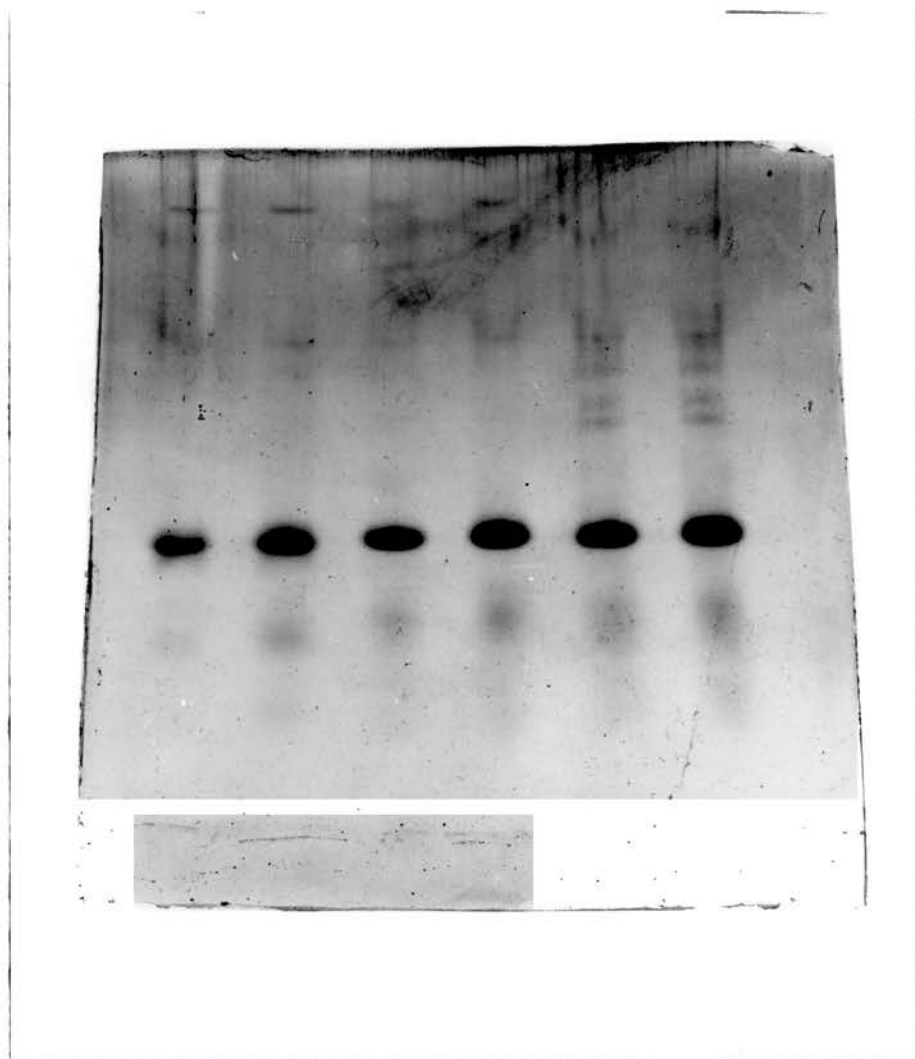


Fig. 22 SDS polyacrylamide gradient gel after electrophoresis of, from L to R (in duplicate), horse spleen apoferritin, human spleen apoferritin and rat liver apoferritin.

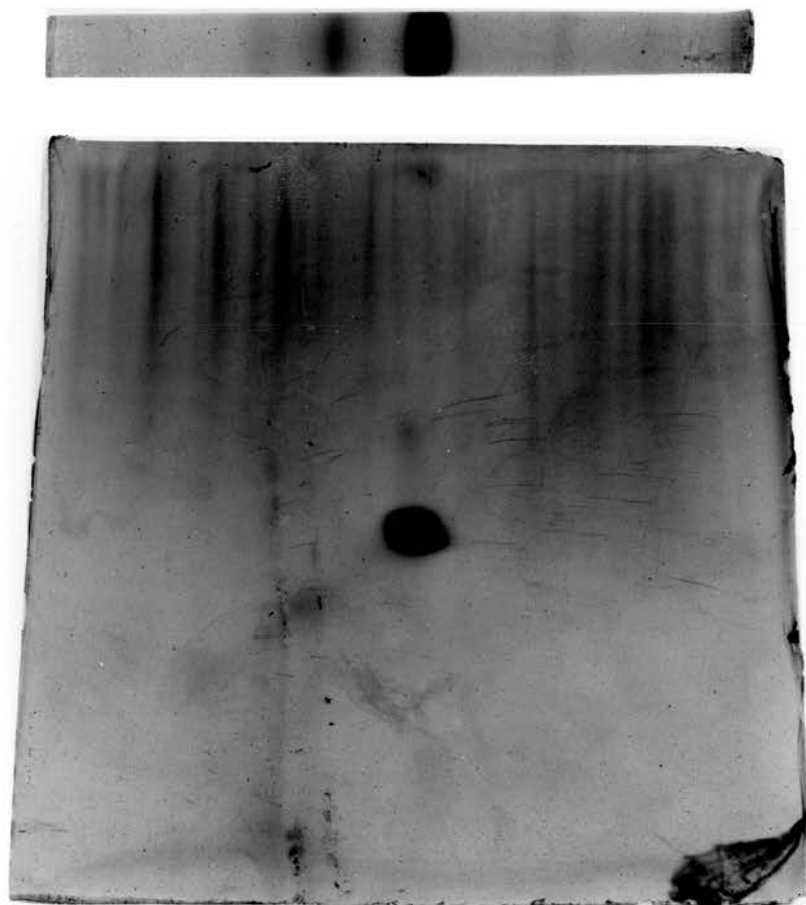


Fig. 23 Polyacrylamide gels after electrophoresis in two dimensions of horse spleen apoferritin.
1st. dimension : SDS electrophoresis
2nd. dimension : SDS polyacrylamide gradient electrophoresis.

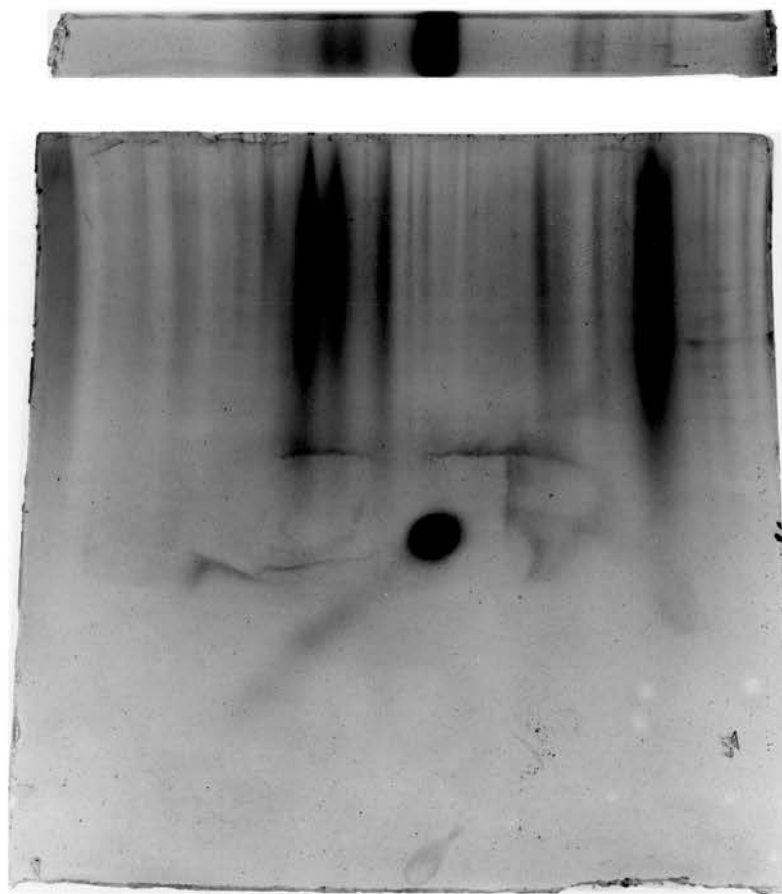


Fig. 24 Polyacrylamide gels after electrophoresis in two dimensions of rat liver apoferritin.
1st. dimension : SDS electrophoresis
2nd. dimension : SDS polyacrylamide gradient electrophoresis.

Fig.23

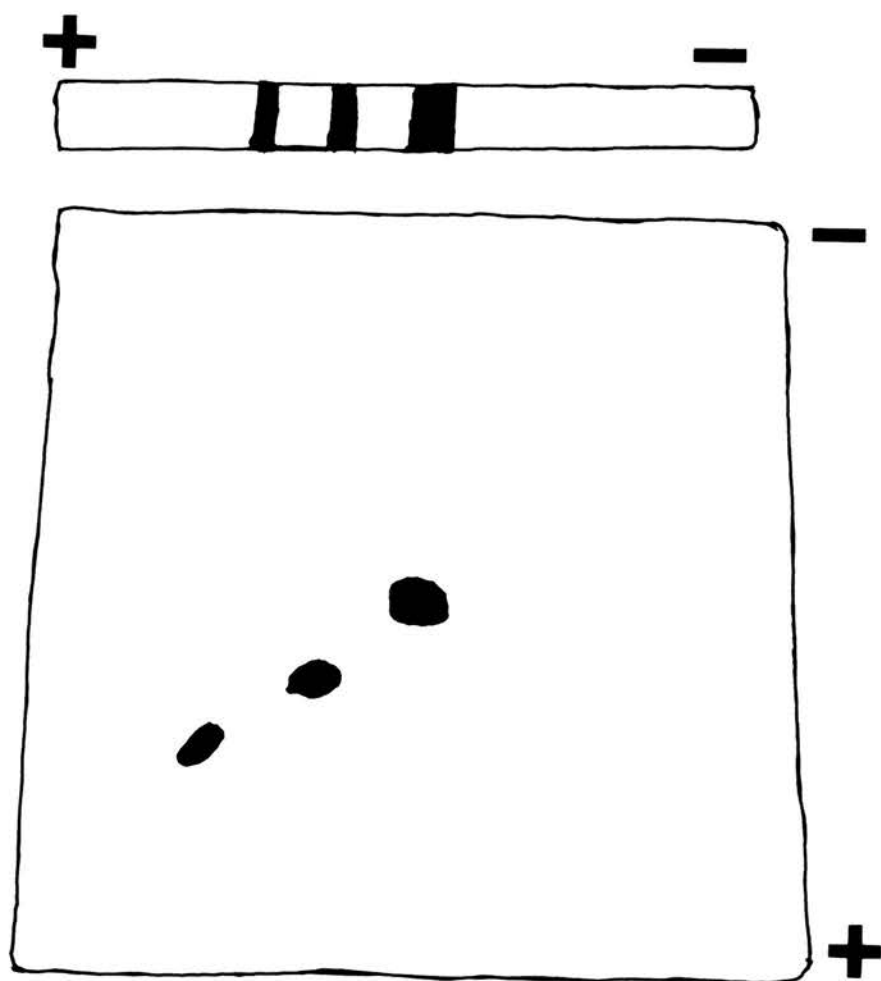
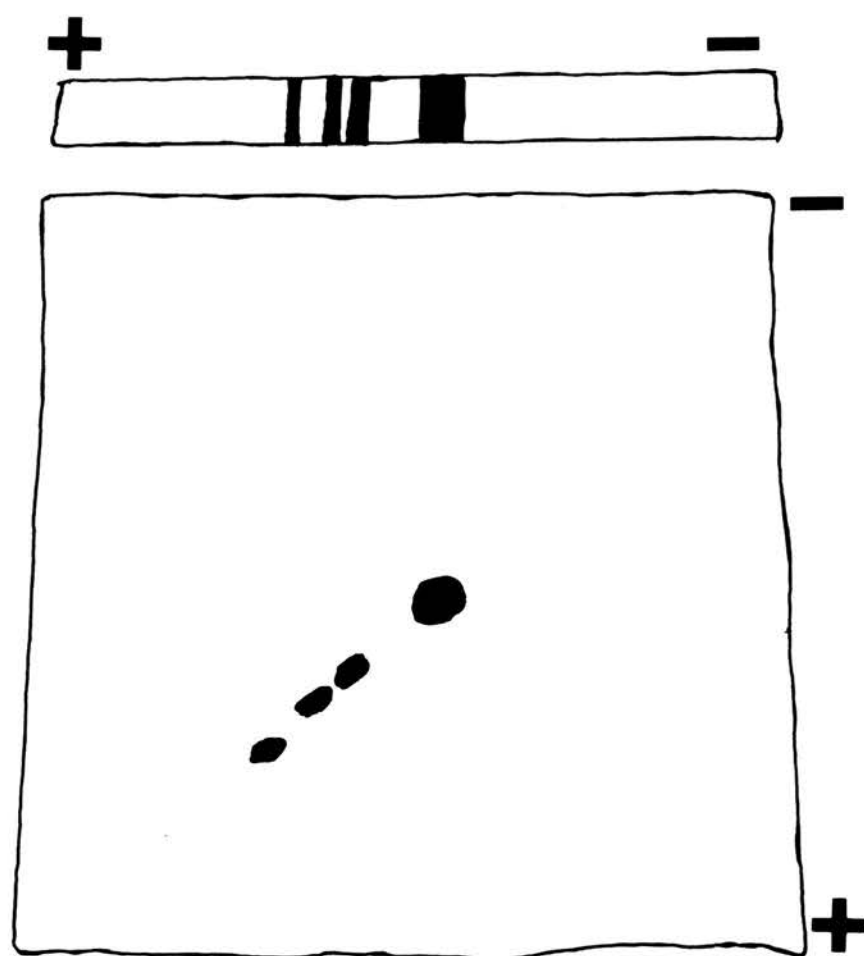


Fig.24



component, and into the typical lower molecular weight components. The rat liver apoferritin sample also showed several faintly staining bands which represented higher molecular weight impurities in that particular preparation.

In the gel shown in Fig. 22, there is no indication whatsoever that the samples might be resolved into components of molecular weight 21,000 and 19,000. This observation seems to oppose claims recently reported (Drysdale *et al.*, 1977) that apoferritin prepared from human tissue could be separated into components of molecular weight 21,000; 19,000 and 15,000 by SDS gradient pore electrophoresis.

Two-dimensional SDS Electrophoresis

Electrophoresis in two dimensions of horse spleen apoferritin is shown in Fig. 23. Three spots can be seen on the second-dimension gel corresponding to the three bands on the first-dimension gel. Since the major band on the first-dimension gel appears as a single spot on the second-dimension gel, this indicates that it represents a single polypeptide rather than a composite of two closely migrating polypeptides of very similar molecular weight. Electrophoresis in two dimensions of rat liver apoferritin (Fig. 24) also showed that the major first-dimension band appeared as a single spot on

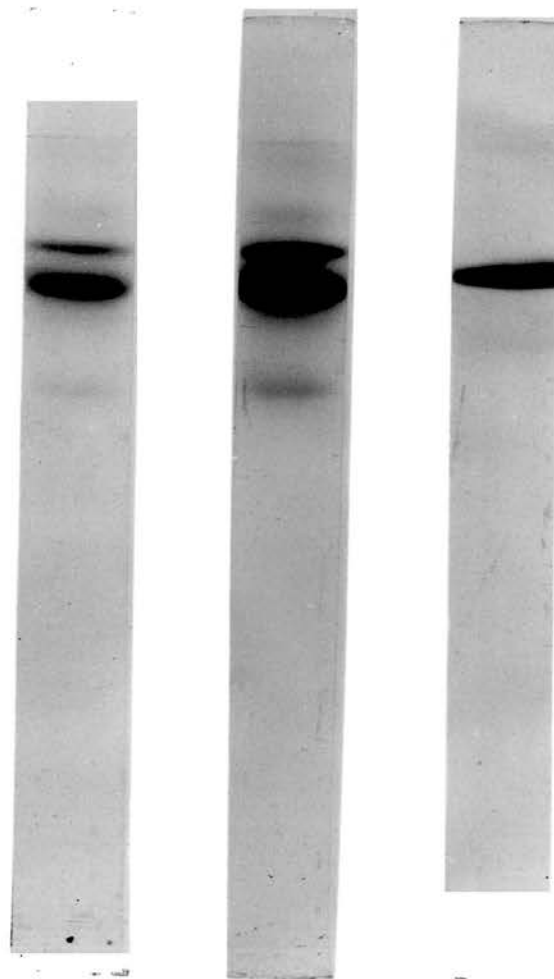


Fig. 25 Acetic acid urea polyacrylamide gels after electrophoresis of, from L to R, human spleen apoferritin, horse spleen apoferritin and rat liver apoferritin.

the second-dimension gel.

Acetic Acid Urea Electrophoresis of Apoferritin

Human spleen, horse spleen and rat liver apoferritins prepared from ferritins isolated by Method 1 (Stanton's method) or by Method 2 (Cardiff method) were subjected to acetic acid urea electrophoresis under the standard conditions described in the methods section. The banding pattern was the same regardless of which method of isolation was employed. Acetic acid urea gels of the three proteins isolated by Method 1 are shown in Fig. 25. The two spleen preparations were identical, with two major bands, and a third less intensely staining more mobile band. The rat liver apoferritin, on the other hand, showed only a single sharp band, the mobility of which coincided with that of the less mobile of the two major spleen bands.

In order to facilitate the description of these and other gels in this section, the bands obtained for the two spleen preparations will be designated c, d and e and that obtained for rat liver c. Two other less mobile bands observed in sample overloaded gels will be designated a and b.

Effect of Reducing Agent

The effect of reducing agent was determined by

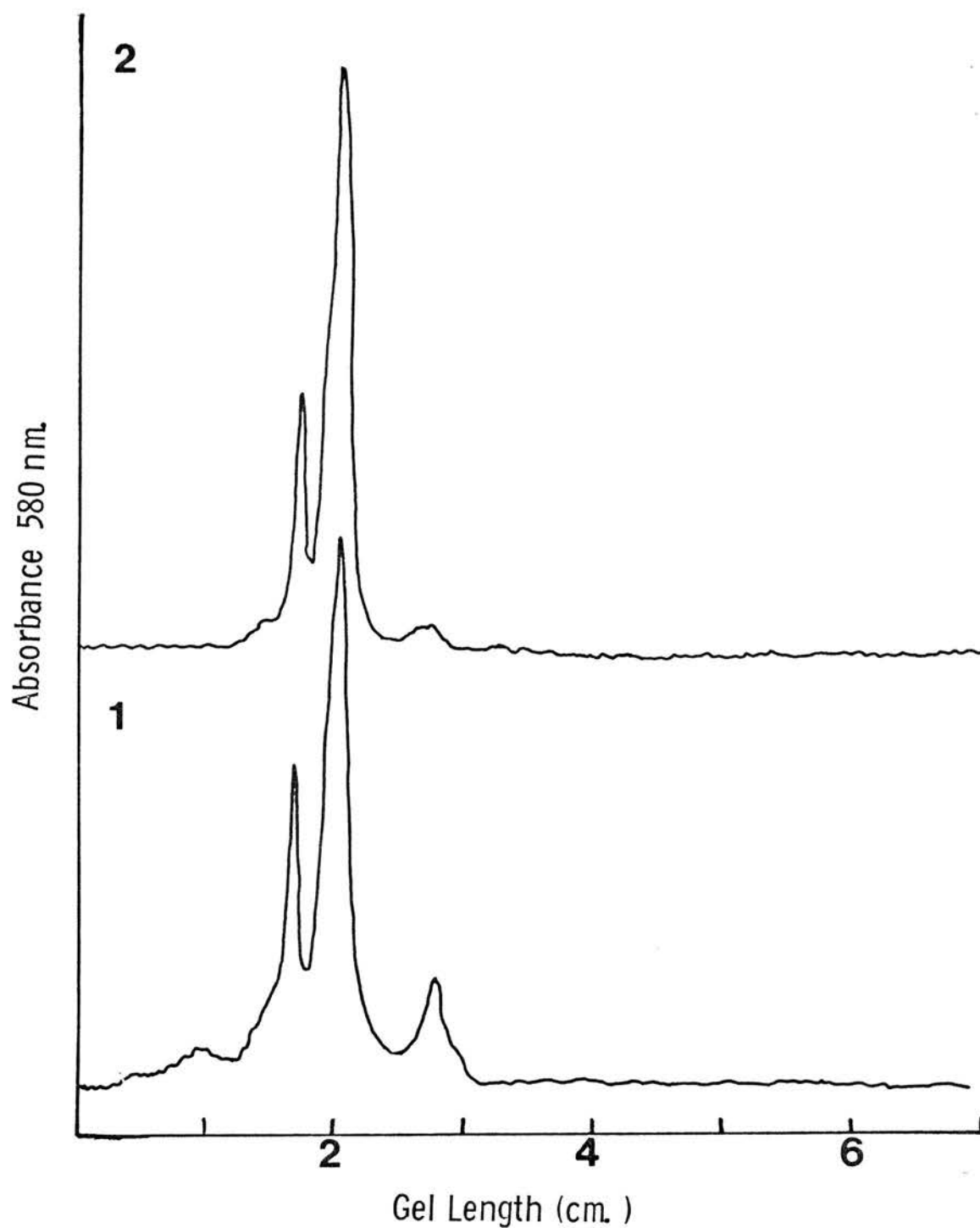


Fig. 26 Densitometric scans of stained acetic acid urea polyacrylamide gels, after electrophoresis of horse spleen apoferritin. Before application to the gel, the sample was treated with:

1) 67% (v/v) acetic acid

2) 67% (v/v) acetic acid containing 1% (v/v) 2-mercaptoethanol.

dissolving horse spleen apoferritin (2 mg/ml) in the dissociating solution (67% (v/v) glacial acetic acid)), which was made 1% (v/v) with respect to β mercaptoethanol. In order to determine any effect on the bands, the gels were overloaded, 25 μ g protein being applied to each gel (Fig. 26). The reducing agent caused a decrease in the staining intensity of the most mobile band (e). This observation confirmed reports by Adelman *et al.* (1975) that the most mobile band on acetic acid urea electrophoresis is eliminated by reducing agent. Results described in the next section provide possible explanations as to how this occurs.

Two-dimensional Electrophoresis Involving Acetic Acid Urea Gels

Two-dimensional electrophoresis involving acetic acid urea electrophoresis in the first dimension was performed in two ways; with either SDS gradient or acetic acid urea gradient pore electrophoresis in the second dimension. Since both of the second-dimension gels should separate components on a molecular weight basis, these experiments would show whether the bands separated on the first-dimension acetic acid urea gel represented polypeptides of similar molecular weight and different charge properties or polypeptides of different molecular weight.

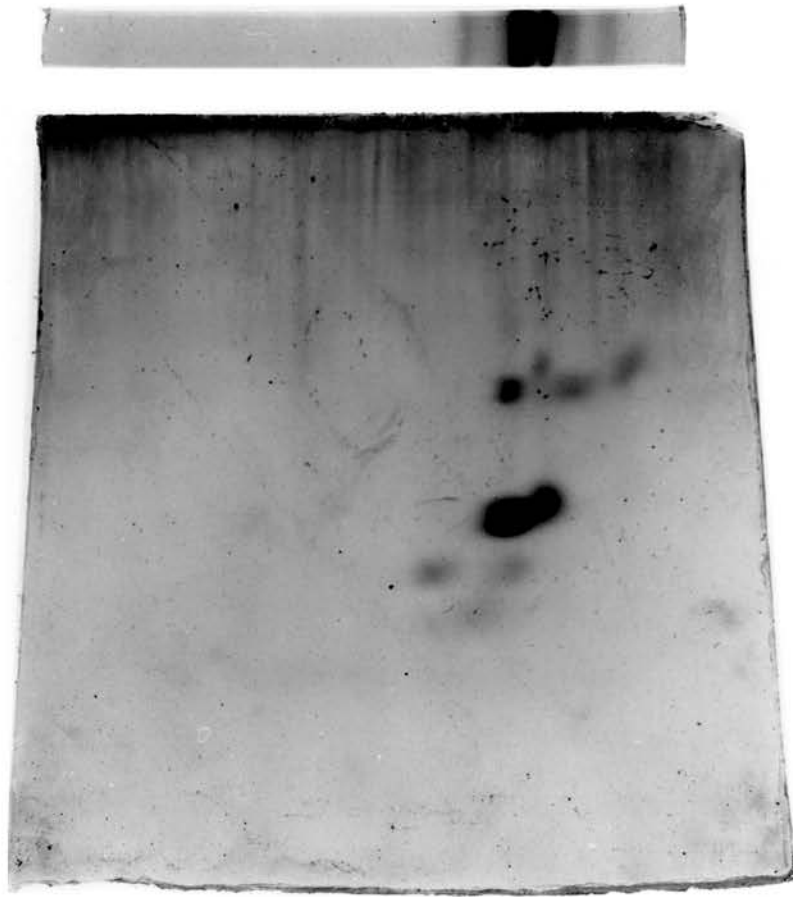


Fig. 27 Polyacrylamide gels after electrophoresis in two dimensions of horse spleen apoferritin.
1 st. dimension : acetic acid urea electrophoresis
2 nd. dimension : SDS polyacrylamide gradient electrophoresis.

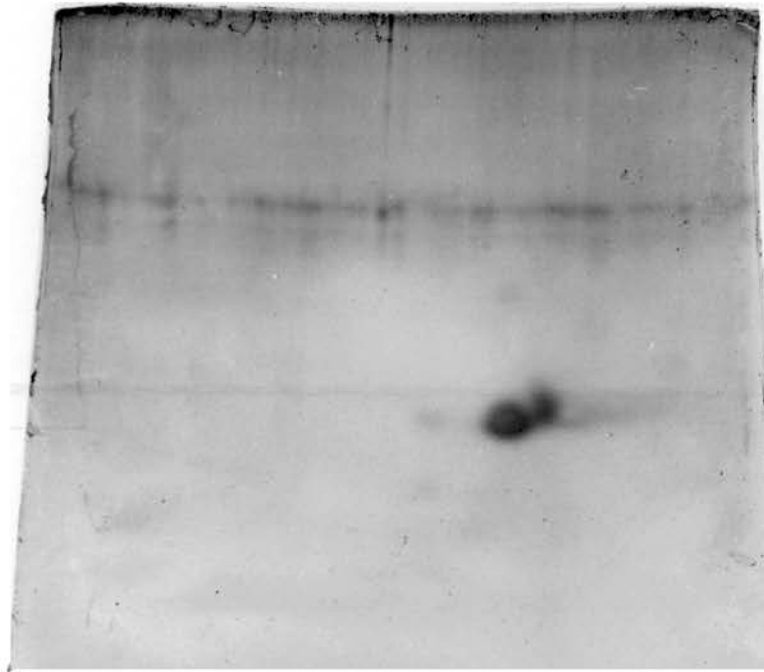


Fig. 28 Polyacrylamide gel after electrophoresis in two dimensions of horse spleen apoferritin.

1st. dimension : acetic acid urea electrophoresis

2nd. dimension (shown here) : SDS polyacrylamide gradient electrophoresis.

Before application to the 2nd. gel, the 1st. dimension gel was treated with reducing agent.

Fig.27

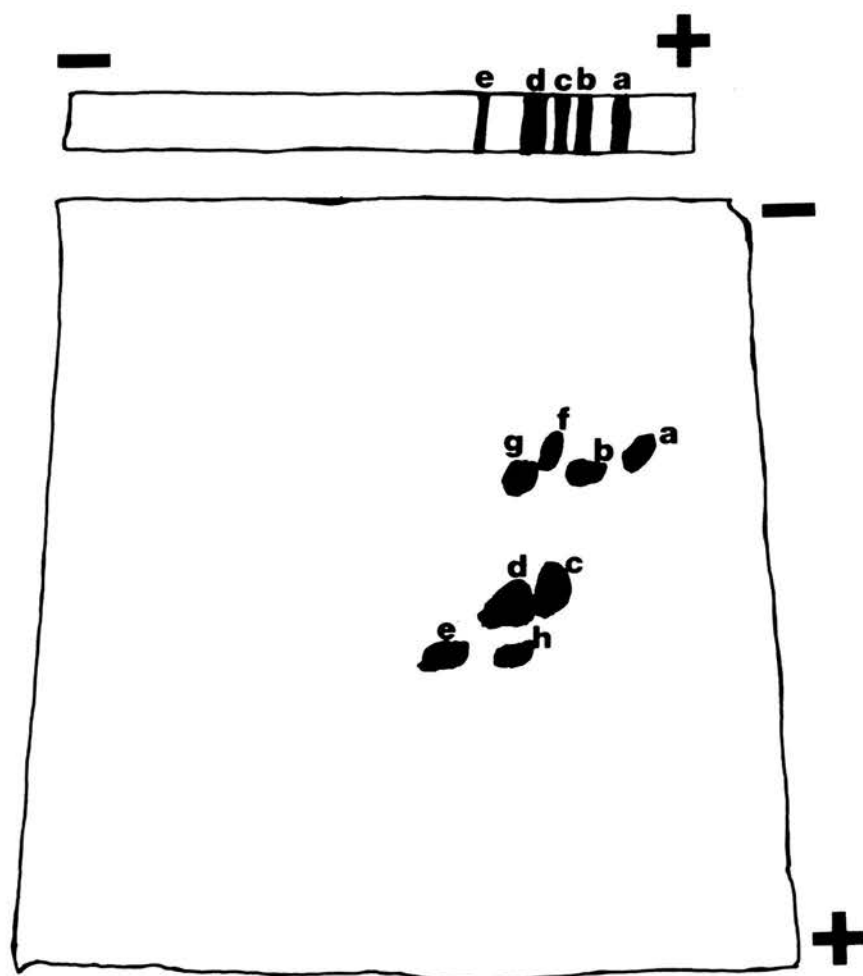
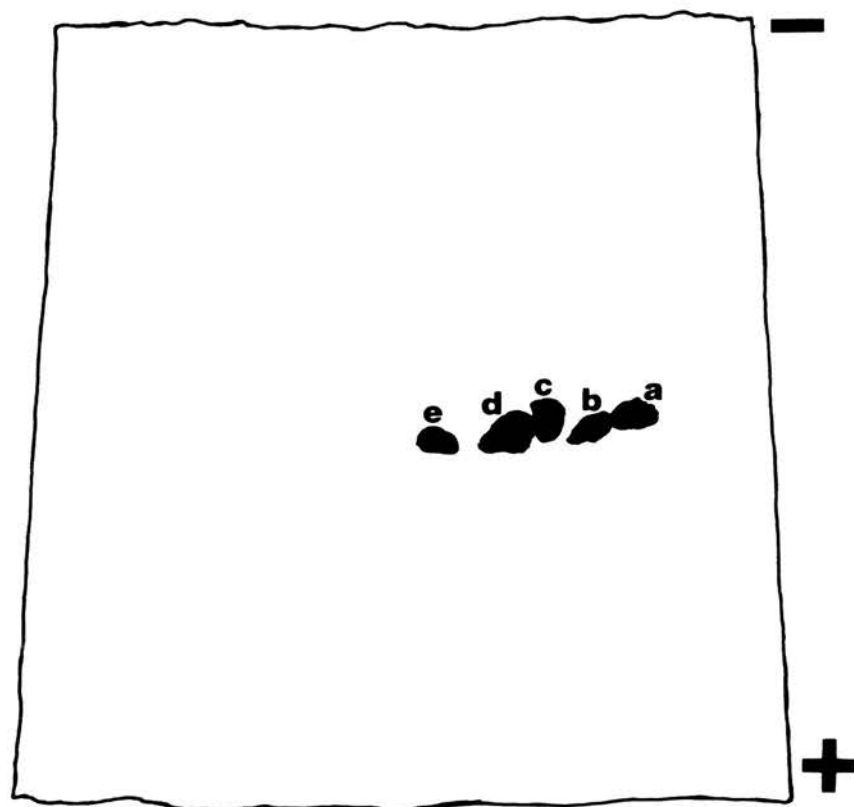


Fig.28



Acetic Acid Urea/Gradient SDS

Two-dimensional electrophoresis of horse spleen apoferritin involving acetic acid urea electrophoresis in a rod gel in the first dimension and SDS gradient pore electrophoresis in the second dimension is shown in Fig. 27. The first-dimension electrophoresis, with excess protein ($\sim 20 \mu\text{g}$) applied to the gel separated the apoferritin into five components; a, b, c, d and e. These components all had different migration rates in the second-dimension gel, which suggested that they represented polypeptides of different molecular weight.

Three other spots were also observed on the second-dimension gel; f, g and h. Spots f and g were derived from the first-dimension bands c and d, and had similar mobilities to spots a and b, while spot h was derived from band d and had the same mobility as spot e. It seems likely from Fig. 27 that spots f and g represent oligomeric species of c and d, thus showing that the first-dimension bands a and b represent oligomers of components c and d. Also, since spot h has the same mobility as spot e, and is derived from component d, then it may be assumed that the first-dimension component e is derived from component d.

The significance of these observations becomes clearer on consideration of Fig. 28, which shows a

second-dimension gradient pore gel of horse spleen apoferritin after first-dimension acetic acid urea electrophoresis. However, before being subjected to electrophoresis in the second-dimension, the first-dimension gel was dialysed against a solution of 0.1% SDS in 0.1 mol/l sodium phosphate buffer pH 7.0 containing 1% β -mercaptoethanol. The effect of reducing agent on electrophoresis in the second dimension, has been to eliminate spots f, g and h and cause spots a and b to migrate to the same extent as spots c and d. This is consistent with the view that the first-dimension components a and b are oligomers of c and d formed by intermolecular disulphide bonding. Furthermore, spot e has now migrated to the same extent as spot d indicating that this component might be formed from intramolecular disulphide bonding within component d, resulting in a polypeptide which behaves on electrophoresis as if it had a smaller molecular weight.

These experiments have confirmed reports by Drysdale *et al.* (1977), on evidence obtained from similar two-dimensional electrophoresis experiments, that the bands seen on acetic acid urea electrophoresis of apoferritin represent polypeptides of different molecular weight. Drysdale *et al.* (1977) further assigned molecular weights of 21,000; 19,000 and 15,000 to these unreduced polypeptides. The gels shown in Figs. 27 and 28 were not

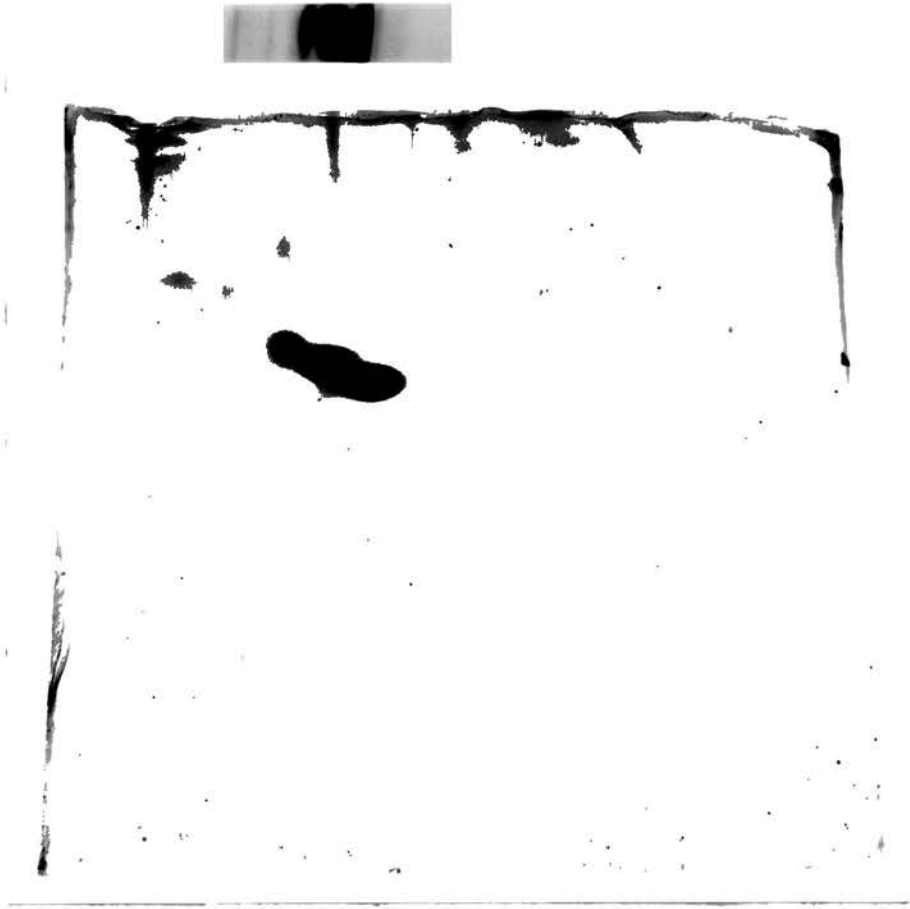


Fig. 29 Polyacrylamide gels after electrophoresis in two dimensions of horse spleen apoferritin and myoglobin.

1st. dimension : acetic acid urea electrophoresis
2nd. dimension : SDS polyacrylamide gradient electrophoresis.

calibrated, and so it was not possible to confirm these molecular weight determinations. The nearest achievement in these studies to a calibration of the two-dimensional electrophoresis is shown in Fig. 29 where myoglobin was run along with horse spleen apoferritin on acetic acid urea electrophoresis in the first dimension. From the relative migration distance of this protein and the apoferritin components in the second-dimension gel, the apparent molecular weight of these three apoferritin components can be estimated as being approximately 19-21,000; 18-19,000 and about 15,000.

Acetic Acid Urea/Gradient Acid Urea

Assuming that the acetic acid urea electrophoretic bands a and b represent oligomeric species and band e an artefact of disulphide bonding, then this system separates horse spleen apoferritin into two closely migrating polypeptides of molecular weight approximately 19,000 and 21,000, while SDS electrophoresis on the other hand shows only a single protein staining band in this molecular weight region. It might appear then that acetic acid urea electrophoresis is a more sensitive method of separating polypeptide chains. However, one could also postulate that these components are artifactual, the 19,000 molecular weight polypeptide being derived from the 21,000 molecular weight polypeptide during acetic acid

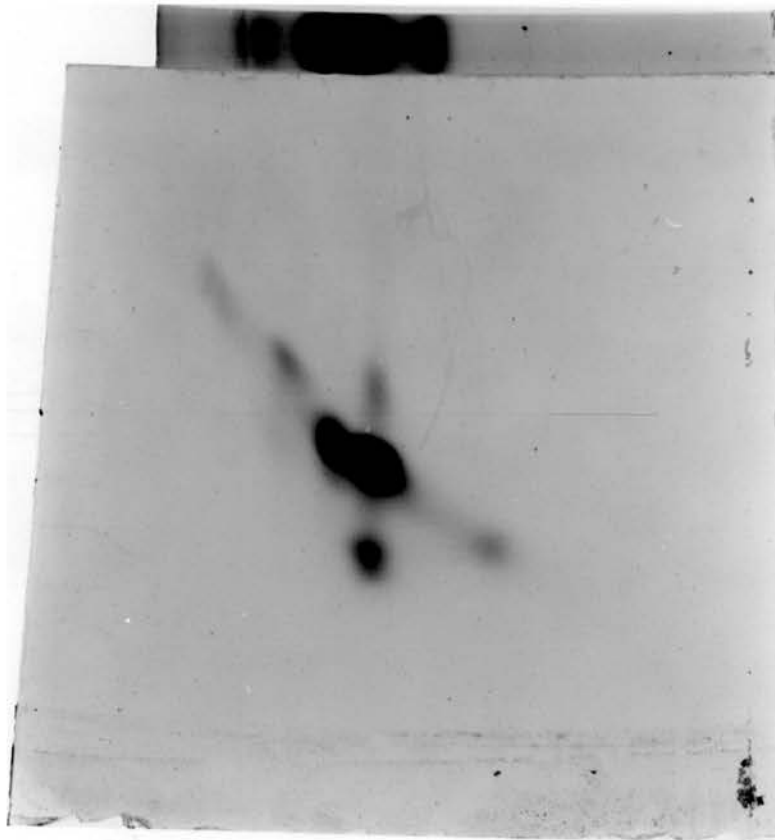


Fig. 30 Polyacrylamide gels after electrophoresis in two dimensions of horse spleen apoferritin.
1st. dimension :acetic acid urea electrophoresis
2nd. dimension : acetic acid urea polyacrylamide gradient electrophoresis.

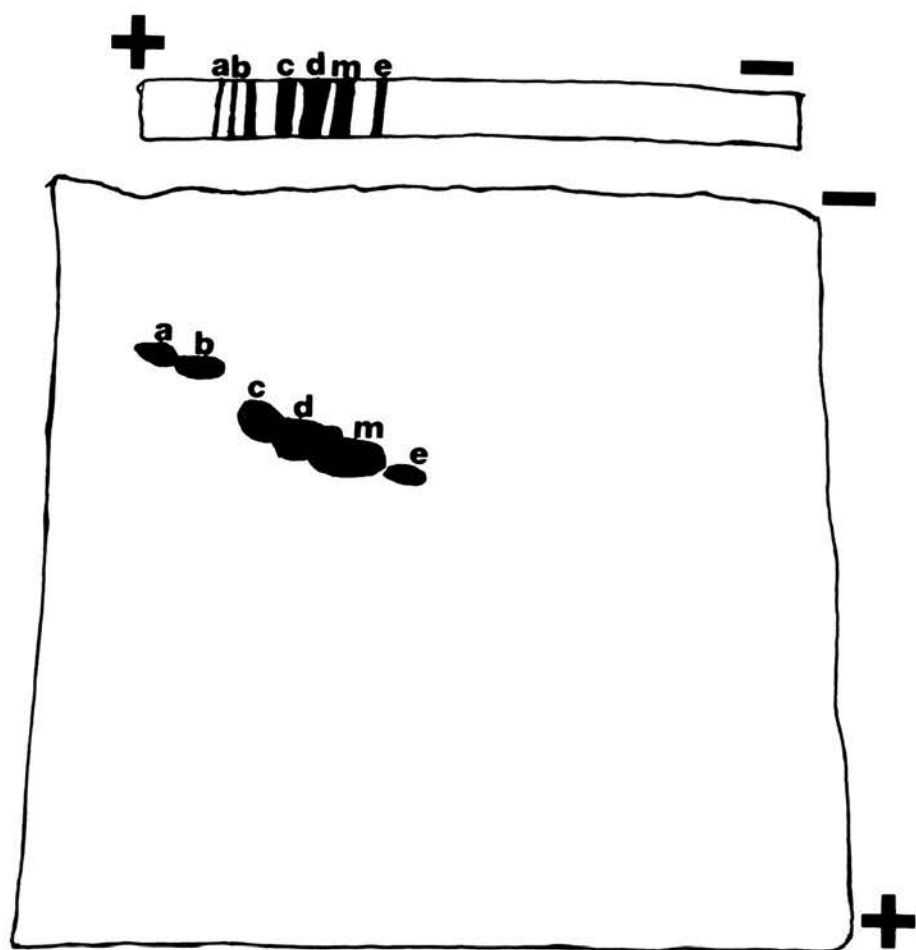


Fig.29

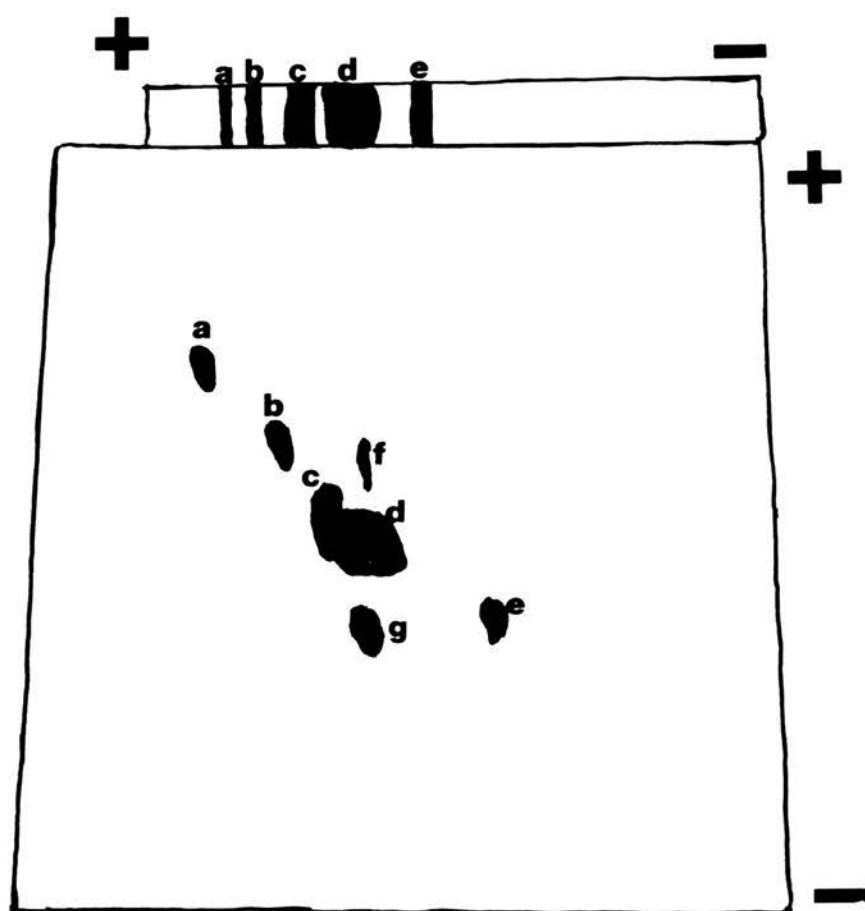


Fig.30

urea electrophoresis either by some form of intramolecular bonding or by "clipping off" a small peptide of molecular weight 2,000.

This theory was tested by subjecting horse spleen apoferritin to electrophoresis in the first dimension on acetic acid urea electrophoresis and in the second dimension on acetic acid urea gradient pore electrophoresis. (Fig. 30). Bands a, b, c, d and e all appeared as single spots on the second-dimension gel, while band d was also resolved into the oligomeric species f and the artefactual species g. In this experiment, no oligomeric species derived from band c was observed on the second-dimension gel.

This experiment has shown that, spots f and g apart, the first-dimension components c and d appeared as single spots on the second-dimension gel thus disputing the theory that component d was derived from component c during electrophoresis, since if this was the case band c would be separated into two spots on the second-dimension gel with mobilities similar to those of spots c and d.

One could follow the above argument further by claiming that in order to observe the conversion of the 21,000 molecular weight component to the 19,000 molecular weight component the whole protein is required,

and that simply subjecting the separated larger component to electrophoresis is not sufficient treatment. This argument however does not seem valid, since in all acetic acid urea electrophoresis experiments, the protein was dissociated into its subunits by prior treatment with 67% acetic acid.

5.4 Conclusion

The results obtained from applying the two types of electrophoresis; SDS and acetic acid urea, and the combination of the two in two-dimensional electrophoresis, to an analysis of the subunit structure of apoferritin, have confirmed many of the previous reports in the literature (Niitsu *et al.*, 1973; Ishitani *et al.*, 1975a); 1975(b); Adelman *et al.*, 1975), while casting doubt on the observations obtained in other laboratories (Linder *et al.*, 1974; Drysdale *et al.*, 1977).

SDS electrophoresis has confirmed for the three apoferritins studied, the presence of multiple bands representing components of apparent molecular weight 18-19,000; "15,000"; 12-13,000 and another below 10,000 which probably has a molecular weight of about 6-7,000. The word apparent is used here, since it has been shown that the band supposedly representing a component of molecular weight 15,000 is in fact artefactual, but to

facilitate description of this band, it will be designated "15,000" m.wt. band, on the basis that its position in the gel is that normally occupied by a polypeptide with a molecular weight of 15,000. This applies also to the treatment of the corresponding band on acetic acid urea gels. Ishitani *et al.* (1975(a)) have shown that the sum of the amino acid composition of the smallest SDS component and the 12-13,000 m.wt component is equal to that of the 18-19,000 m.wt component. On this basis, it seems likely that 6-7,000 is a good approximation of its molecular weight.

In rat liver apoferritin, a 15,000 m.wt band was observed in gels even after the sample was treated with β mercaptoethanol, in a quantity which was sufficient to reduce the corresponding component present on gels of the two spleen apoferritins. The implications of this will be discussed in greater detail later.

It was not possible either by electrophoresis in SDS homogeneous concentration gels, SDS gradient pore gels or two-dimensional SDS electrophoresis to separate apoferritin isolated from any of the tissues used, into two polypeptides of molecular weight 19,000 and 21,000 as reported by Drysdale *et al.* (1977).

Acetic acid urea electrophoresis on the other hand,

resolved both horse and human spleen apoferritin into three components, which on second-dimension SDS gradient pore gels were shown to have approximate molecular weights of 20-21,000; 18-19,000 and "15,000", thus confirming similar claims by Drysdale *et al.* (1977). The smallest component was shown to be formed by intramolecular disulphide bonding within the 18-19,000 molecular weight component. This leads to the speculation that the "15,000" m.wt. band observed on SDS electrophoresis is formed in the same way. Also present on sample overloaded acetic acid urea gels, were two slow migrating components which were shown to occur as a result of intermolecular disulphide bonding between the two main components.

Just as on SDS electrophoresis, rat liver apoferritin behaved differently from the two spleen apoferritins, then so too on acetic acid urea electrophoresis rat liver apoferritin showed only one protein staining band the mobility of which coincided with that of the 20-21,000 molecular weight component of the spleen apoferritins.

Finally, the actual relationship which the electrophoretic results described above, and the gel filtration studies described in the previous chapter bear to each other, will be approached experimentally in the following chapter.

CHAPTER VI

THE RELATIONSHIP BETWEEN THE POLYPEPTIDE COMPONENTS OF APOFERRITIN OBTAINED BY THE VARIOUS METHODS OF DISSOCIATION SEPARATION AND ANALYSIS

Table 4. Summary of the Results Described in Chapters 4 and 5

Concerning the Products of Dissociation of Apoferritin

| Tissue Source | Method of Dissociation | Method of Analysis | Apparent m.wt. $\times 10^{-3}$ |
|---------------|---------------------------|-----------------------|------------------------------------|
| Human Spleen | Guanidine HCl | Gel filtration | 18.5; 12-13; <10 |
| | Acetic acid | Gel filtration | 18-19 |
| | Acetic acid | Electrophoresis | 21; 19 |
| | SDS | Electrophoresis | 18.5; 12-13; 6-7 |
| Horse Spleen | Guanidine HCl | Gel filtration | 18.5; 12-13; <10 |
| | Acetic acid | Gel filtration | 18-19 |
| | Acetic acid | Electrophoresis | 21; 19 |
| | SDS | Electrophoresis | 18.5; 12-13; 6-7 |
| Rat Liver | Guanidine HCl | Gel filtration | 19; 12-13; <10 |
| | Acetic acid | Gel filtration | 19 |
| | Acetic acid | Electrophoresis | 21 |
| | SDS | Electrophoresis | 18.5; 15; 12-13; 6-7. |

6.1 Introduction

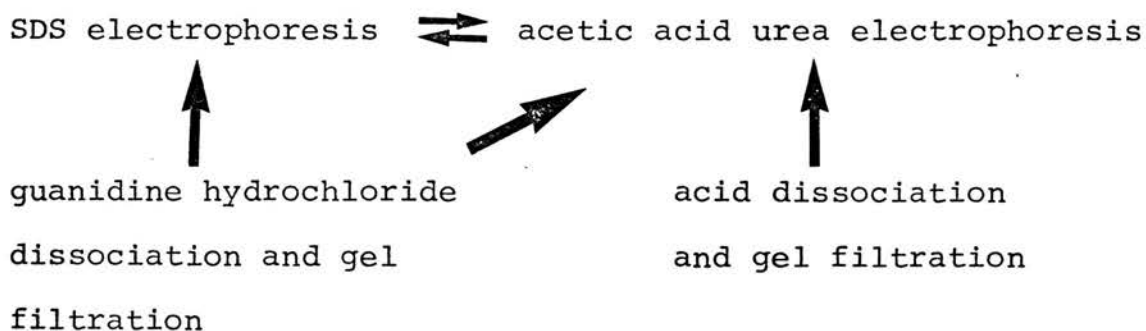
As described in Chapters 4 and 5, the number and size of polypeptide components obtained when apoferritin was dissociated, depended on the dissociating agent and method of analysis employed. The main features of the analysis on the products of dissociation of apoferritin are summarised in Table 4 and include:

- (i) the identification of polypeptides of molecular weight 18-19,000; 12-13,000 and 6-7,000 on both SDS electrophoresis and gel filtration in the presence of guanidine hydrochloride
- (ii) the absence of any 12-13,000 and 6-7,000 molecular weight polypeptides on acetic acid urea electrophoresis and gel filtration in acid buffer.
- (iii) the identification of a polypeptide with an approximate molecular weight of 21,000 on acetic acid urea electrophoresis, which is not observed by any other method of analysis.
- (iv) the early elution from Sephadex G-100 of the largest polypeptide of rat liver apoferritin dissociated in either guanidine hydrochloride or 67% acetic acid, which indicated that this polypeptide is larger than the equivalent

human or horse spleen polypeptides. However this observation was not supported by SDS electrophoresis, where the main rat liver apoferritin component had a migration rate similar to that of the two spleen polypeptides.

A noticeable trend is the similarity in the results obtained for SDS electrophoresis, gel filtration in guanidine hydrochloride and gel filtration in acid, in so far as the largest polypeptide observed has a molecular weight of 18-19,000. On the other hand, the acetic acid urea electrophoretic analysis is the only technique which shows a component of molecular weight about 21,000.

With these technical inconsistencies in mind, experiments were designed, whereby polypeptide components of apoferritin separated by one technique would be subjected to analysis by one or more of the other techniques. An outline of the intended cross-checks is shown below.



The carboxymethylated and uncarboxymethylated polypeptides of apoferritin dissociated and separated by gel filtration in the presence of guanidine hydrochloride were subjected to SDS electrophoresis and acetic acid urea electrophoresis. Similarly the products of 67% acetic acid dissociation, separated by gel filtration in acid buffer, were subjected to acetic acid urea electrophoresis. It was not possible to subject these latter separated fractions to SDS electrophoresis, since both the SDS binding reaction and electrophoresis were carried out at neutral pH, and raising the pH of the acid dissociated polypeptide fractions would cause them to reassociate to form whole apoferritin.

The products of acetic acid urea electrophoresis were subjected to SDS gradient pore electrophoresis in the second dimension. These results have already been described in Chapter 5, but because of their relevance to these comparative studies, will be summarised in this chapter. A further experiment undertaken, was to subject the products of SDS electrophoresis to electrophoresis in the second dimension on acetic acid urea gradient gels. Before the separated polypeptides would migrate towards the cathode in the second-dimension gel, it was necessary to remove the bound negatively charged SDS molecules. The method employed to separate SDS from the protein was based on that of Maizel (1971), and involved incubating

the first-dimension gel at 37°C in 8 mol/l urea to weaken the protein-SDS interactions. Although this experiment was repeated three times, it was unsuccessful since the protein never migrated from the SDS gel into the second-dimension gel. Confirmation of this was obtained by the appearance of protein staining bands on the SDS gel after the second dimension electrophoresis. It would appear that treating the SDS gel with 8 mol/l urea was not sufficient to break the hydrophobic bonds between the SDS and the polypeptides.

All the methods employed in the experiments reported in this chapter have been previously described in chapters 3, 4 and 5.

6.2 Results

Analysis by SDS Electrophoresis of the Products Dissociated in Guanidine Hydrochloride

Apoferritins, prepared from ferritin isolated from horse spleen, human spleen and rat liver, were dissociated in 7 mol/l guanidine hydrochloride, and subjected to gel filtration on Sephadex G-100 equilibrium in 7 mol/l guanidine hydrochloride, as described in chapter 4. In one experiment, the samples were carboxymethylated, and in another they were applied to the column without prior

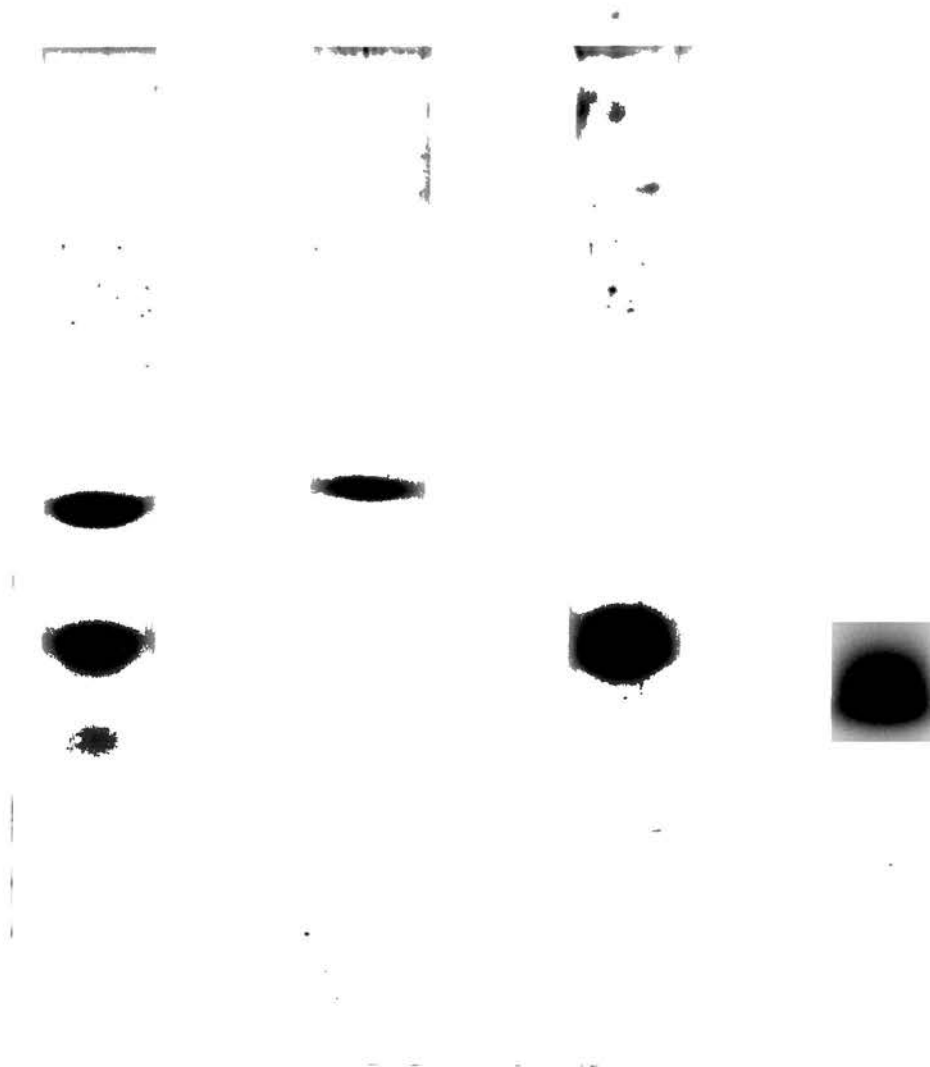


Fig. 31 SDS polyacrylamide gels after electrophoresis of, from L to R, horse spleen apoferritin, and the three main components of horse spleen apoferritin eluted from Sephadex G-100 equilibrated in 7 Mol/L guanidine HCl.

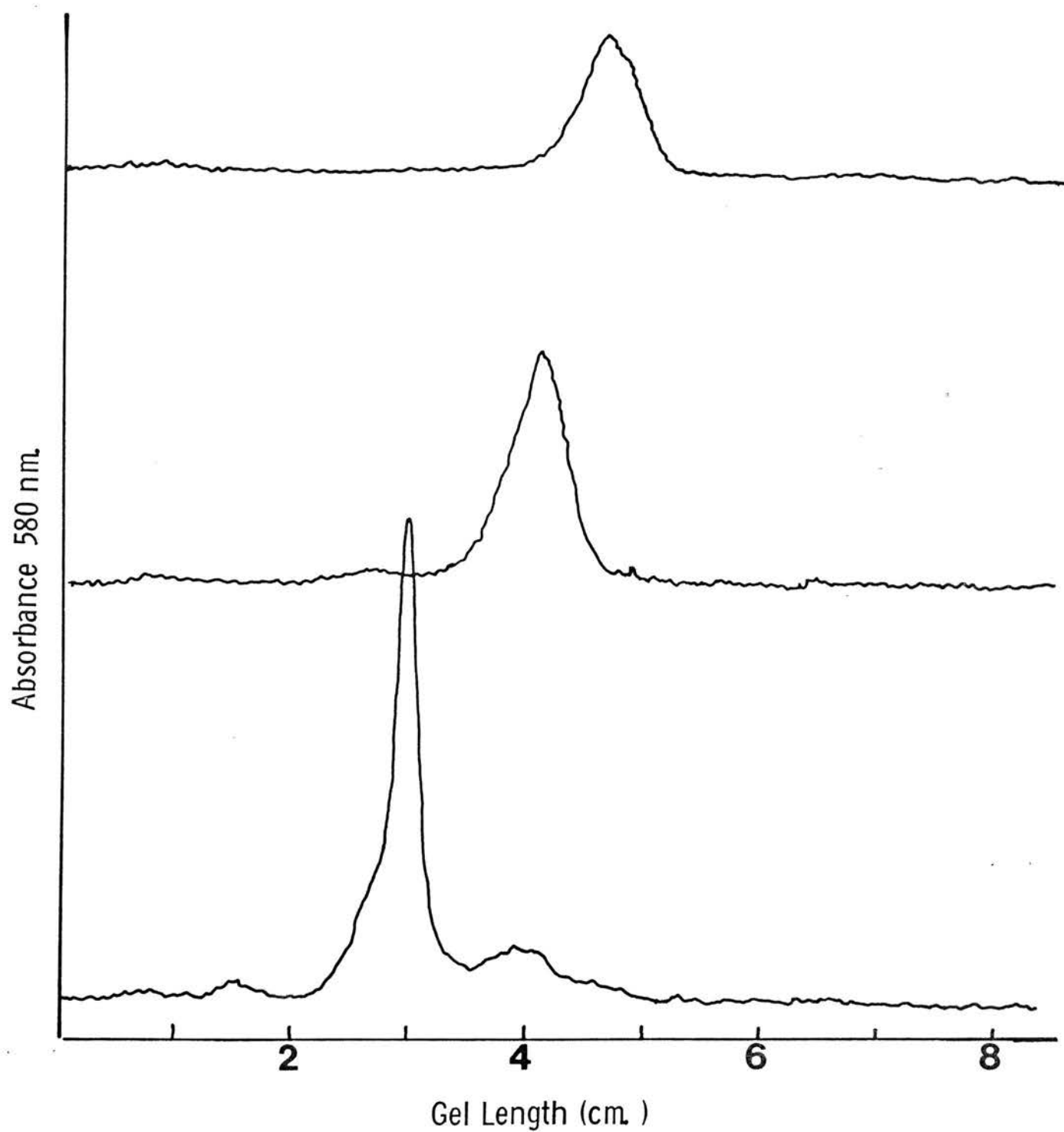


Fig. 32 Densitometric scans of stained SDS polyacrylamide gels, after electrophoresis of the three components of human spleen apoferritin eluted from Sephadex G-100 in the presence of 7 Mol/L guanidine HCl.

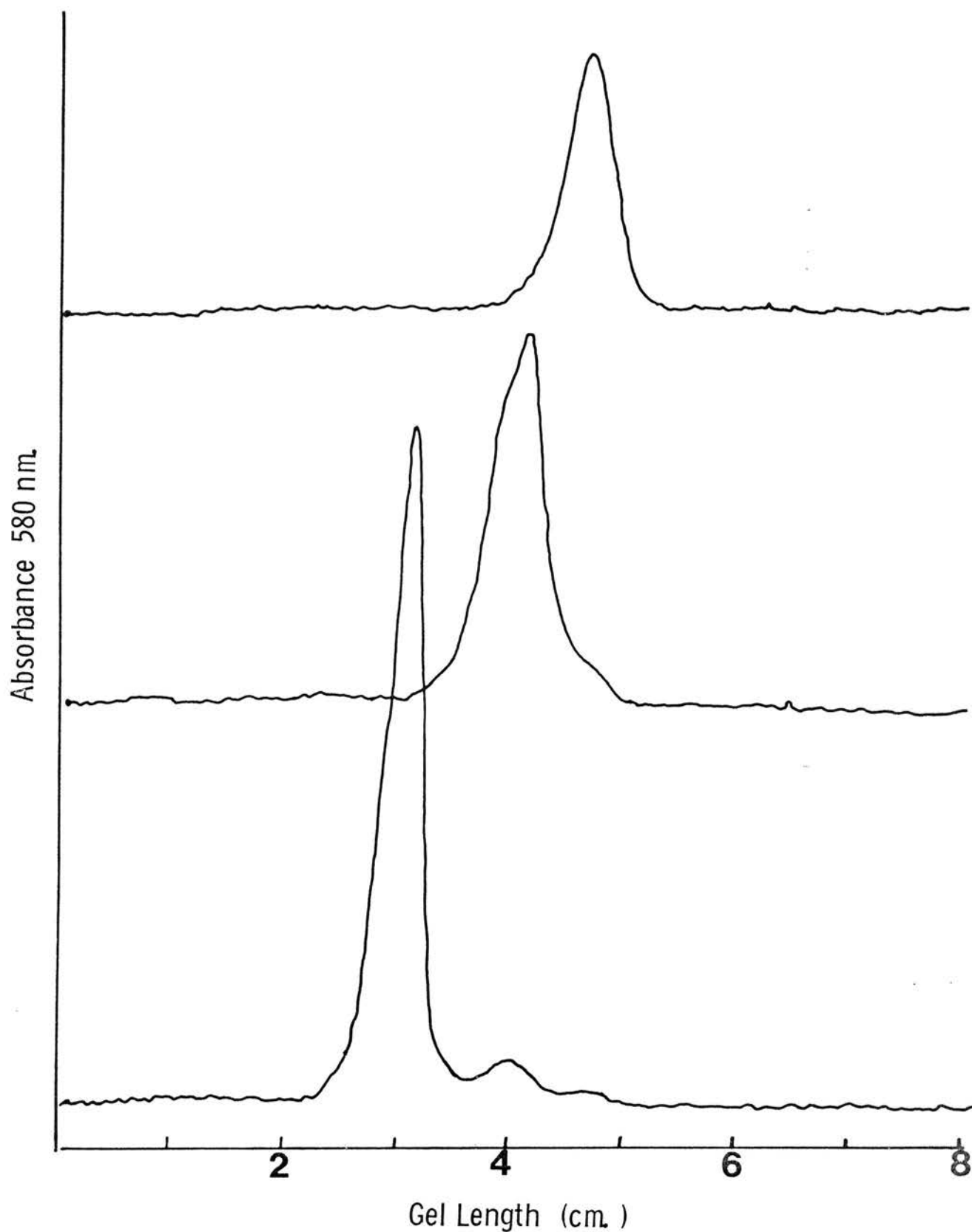


Fig. 33 Densitometric scans of stained SDS polyacrylamide gels, after electrophoresis of the three components of rat liver apoferritin eluted from Sephadex G-100 in the presence of 7 Mol/L guanidine HCl.

carboxymethylation. Fractions corresponding to each of the eluted components were collected, dialysed against distilled water and the precipitated polypeptides lyophilised and subjected to SDS electrophoresis.

SDS PAGE gels of horse spleen apoferritin, and the three polypeptides eluted from Sephadex G-100 after previously blocking the protein's sulfhydryl groups by carboxymethylation are shown in Fig. 31. The apoferritin sample was separated into the characteristic three components, while the eluted guanidine hydrochloride dissociated polypeptides had the same electrophoretic mobilities as the corresponding SDS-dissociated polypeptides. Scans of the stained SDS gels of the polypeptides, separated by gel filtration in guanidine hydrochloride, of human spleen and rat liver apoferritin (Figs. 32 & 33) show that as for horse spleen apoferritin, these components appeared as bands representing polypeptides of molecular weight 18-19,000; 12-13,000 and "6-7,000". One could conclude from this experiment that the three polypeptides separated by dissociation and gel filtration in guanidine hydrochloride are identical to those separated by dissociation and electrophoresis on polyacrylamide gels in the presence of SDS.

Gels obtained by SDS electrophoresis of the polypeptides separated by gel filtration in the presence of 7 mol/l guanidine hydrochloride without prior carboxy-

methylation of the sample gave results identical to these for the carboxymethylated polypeptides. The negative charge on the protein bound SDS molecules would appear to "swamp out" any change in the net charge on the polypeptide due to the carboxymethyl group. As a result, the carboxymethyl group does not affect the electrophoretic properties of the separated polypeptides.

Analysis by Acetic Acid Urea Electrophoresis of the
Products Dissociated in Guanidine Hydrochloride

As for the experiments described in the previous section, the polypeptides of the three apoferritins studied were separated by dissociation and gel filtration in the presence of guanidine hydrochloride after previously subjecting the sample to carboxymethylation, and in the absence of carboxymethylation. The separated polypeptides were then subjected to acetic acid urea electrophoresis. In order to facilitate the description of these experiments, each protein will be dealt with separately. Also, for reasons explained later only the experiments on the 18-19,000 molecular weight polypeptide will be described here.

(a) Horse Spleen Apoferritin

The acetic acid urea gels and the corresponding

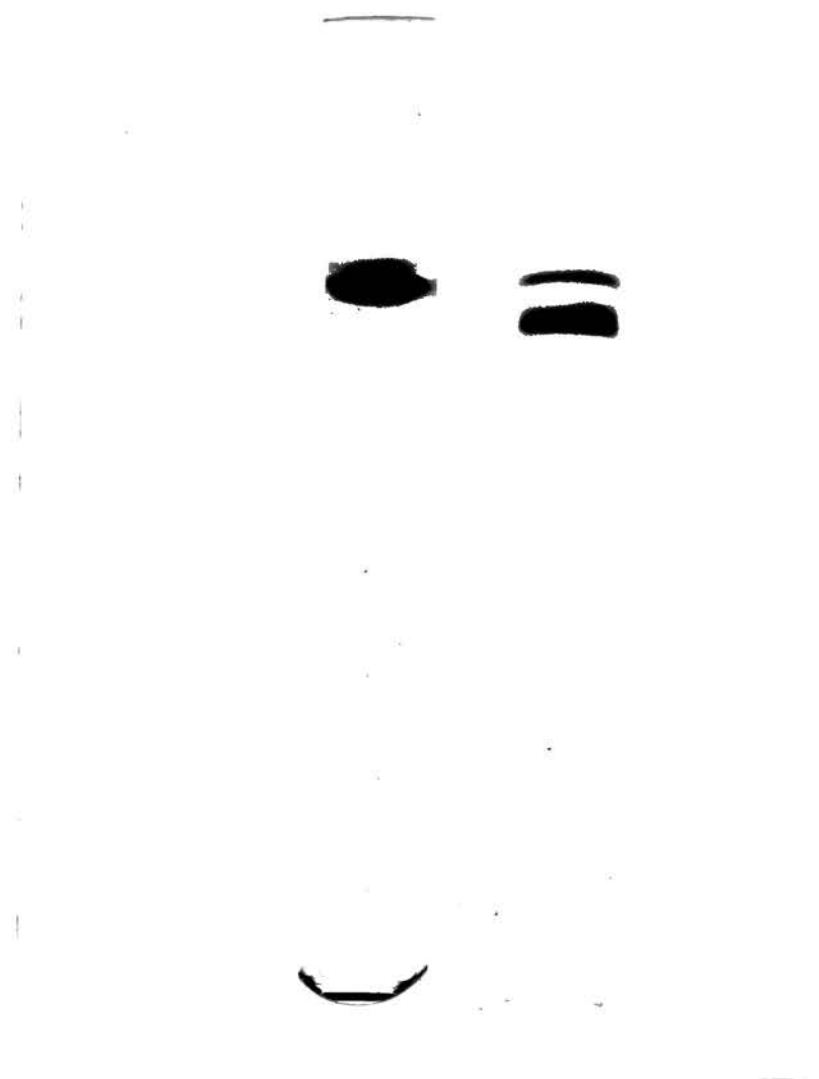


Fig. 34 Acetic acid urea polyacrylamide gels after electrophoresis of, from R to L, horse spleen apoferritin, and the carboxymethylated main component of horse spleen apoferritin eluted from Sephadex G-100 equilibrated in 7 Mol/l guanidine HCl.

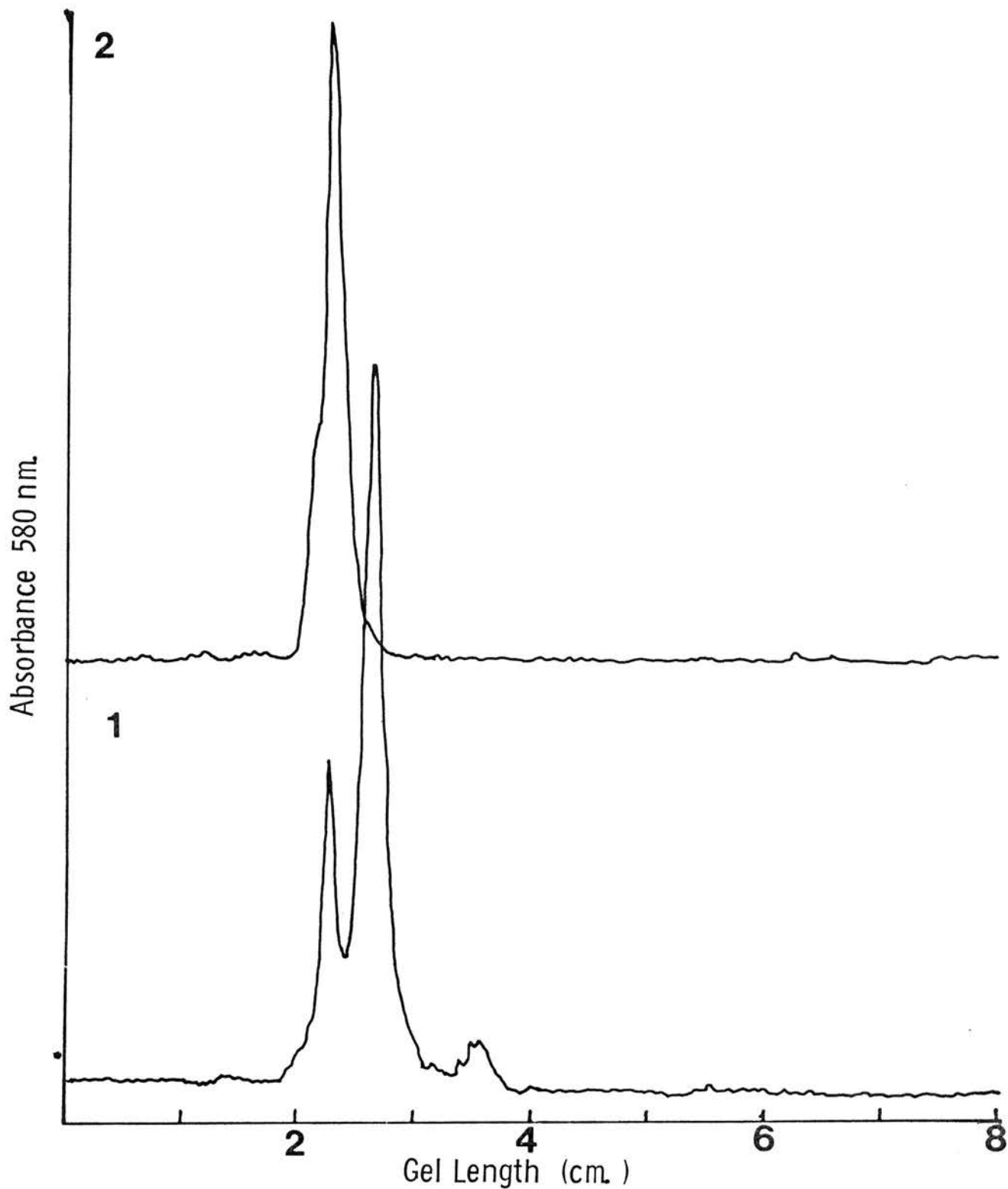


Fig. 35 Densitometric scans of stained acetic acid urea polyacrylamide gels, after electrophoresis of:
1) horse spleen apoferritin
2) the main carboxymethylated component of horse spleen apoferritin eluted from Sephadex G-100 in the presence of 7 Mol/L guanidine HCl.

scans, of horse spleen apoferritin and the 18-19,000 molecular weight polypeptide eluted, after carboxymethylation, from Sephadex G-100 equilibrated in guanidine hydrochloride are shown in Figs. 34 & 35. The apoferritin sample was resolved into the typical three bands, but the guanidine hydrochloride polypeptide showed only two bands. The RF values, calculated as the ratio of the migration distance to the gel length, were as follows:

| Horse Spleen Apoferritin | RF | Horse Spleen Apoferritin Polypeptide | RF |
|-----------------------------|-------|--|-------|
| Band 1 | 0.28 | Band 1 | 0.275 |
| Band 2 | 0.33 | Band 2 | 0.288 |
| Band 3 | 0.375 | | |

It is possible that Bands 1 and 2 on each gel represent the same polypeptides, and that the decreased mobility of the polypeptide components dissociated in guanidine hydrochloride could be due to prior carboxymethylation of the sulfhydryl groups, resulting in a decrease in the net positive charge on the polypeptides, and hence their slower migration towards the cathode. The absence of the most cathodic band in gel (a) can be explained, since it has been shown that this band is eliminated in the presence of reducing agent (Fig. 26), and so has been assumed to be

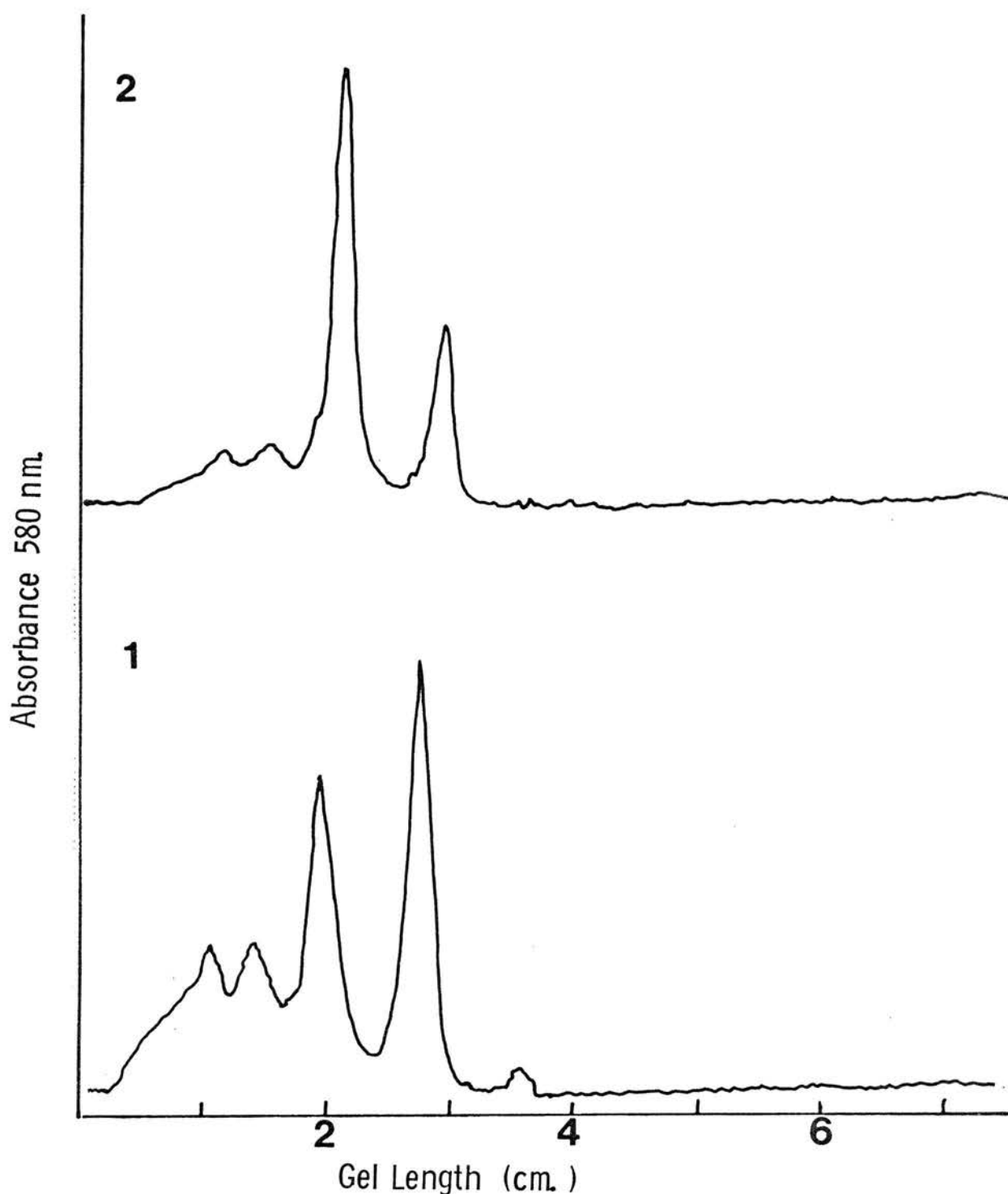


Fig. 36 Densitometric scans of stained acetic acid urea polyacrylamide gels, after electrophoresis of:
1) the main uncarboxymethylated component of horse spleen apoferritin eluted from Sephadex G-100 in the presence of 7 Mol/L guanidine HCl
2) the same sample treated with 1%(v/v) 2-mercaptoethanol , before application to the gel.

formed through disulphide bonding. Consequently if the sulfhydryl groups are protected by carboxymethyl groups no disulphide bonding can occur.

A similar experiment was carried out on the polypeptide obtained without prior carboxymethylation. In this experiment, since the sulfhydryl groups were "free" the effect of reducing agent was determined (Fig. 36). In the absence of reducing agent four protein staining bands were observed:

- (a) two slowly running components which were probably oligomers of the main dissociated polypeptides.
- (b) two more intensely staining bands representing components whose migration rate was similar to that of the 19,000 and "15,000" bands previously described (Chapter 5). The "15,000" component stained more intensely than the 19,000. Although not clearly illustrated in Fig. 36 the component of approximate molecular weight 19,000 appeared visually to consist of two closely migrating bands. It could be due to the presence of the oligomeric bands that this 21,000 molecular weight component does not appear so sharply in Fig. 36.

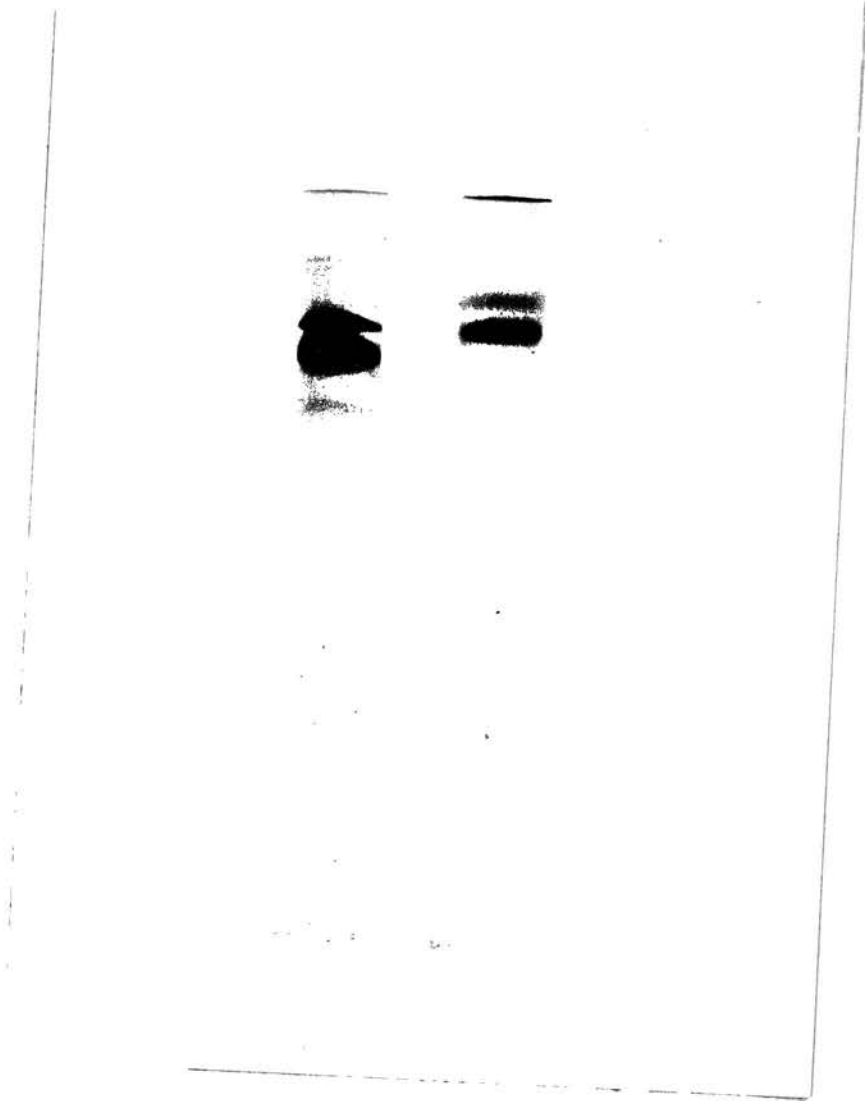


Fig. 37 Acetic acid urea polyacrylamide gels after electrophoresis of, from L to R, human spleen apoferritin, and the carboxymethylated main component of human spleen apoferritin eluted from Sephadex G-100 equilibrated in 7 Mol/L guanidine HCl.

When the sample was treated with 1% (v/v) β mercapto-ethanol, the two slowly moving bands almost completely disappeared, and the staining intensity of the 19,000 molecular weight component increased at the expense of the "15,000". It would appear then that, as occurs with the whole protein (Chapter 5), when the sulfhydryl groups of this isolated polypeptide are unprotected, disulphide bonding occurs giving rise to this artefactual "15,000" component. It seems likely that this is intramolecular disulphide bonding (5.3). A certain amount of inter-molecular disulphide bonding also occurs resulting in the slow moving bands which are eliminated by reducing agent.

(b) Human Spleen Apoferritin

The 18-19,000 molecular weight component of human spleen apoferritin eluted from Sephadex G-100 after dissociation in 7 mol/l guanidine hydrochloride following previous carboxymethylation, and in the absence of carboxymethylation gave a banding pattern on acetic acid urea electrophoresis similar to that given by the corresponding polypeptide from horse spleen apoferritin. An exception was that the decrease in mobility caused by carboxymethylation was more pronounced with the polypeptide from human spleen (Fig. 37). One possible explanation for this is that this latter polypeptide



Fig. 38 Acetic acid urea polyacrylamide gels after electrophoresis of, from L to R, rat liver apoferritin, and the carboxymethylated main component of rat liver apoferritin eluted from Sephadex G-100 equilibrated in 7 Mol/L guanidine HCl.

binds more carboxymethyl groups than that from horse spleen. A comparison of the amino acid content of the respective polypeptides (Crichton, 1973) shows that both contain the same amount of cysteine residues (two), but that the polypeptide from human spleen has seven histidine residues as opposed to the six histidine residues of that from horse spleen apoferritin. As reported by Bryce and Crichton (1973) treatment of horse spleen apoferritin with iodoacetic acid resulted in carboxymethylation of two cysteine residues and one histidine residue per polypeptide. Hence the extra histidine residue in the polypeptide from human spleen might bind an extra carboxymethyl group, and a slower migration rate towards the cathode on acetic acid urea electrophoresis could result.

(c) Rat Liver Apoferritin

The acetic acid urea electrophoresis gels of rat liver apoferritin and the main polypeptide eluted from Sephadex G-100 following dissociation in 7 mol/l guanidine hydrochloride and carboxymethylation are shown in Fig. 38. Both samples appear as a single band with a very faintly staining slow moving component in the right hand gel. The RF value of the "apoferritin" band was 0.246 and that of the polypeptide 0.240. Carboxymethylation has reduced very slightly the migration distance of

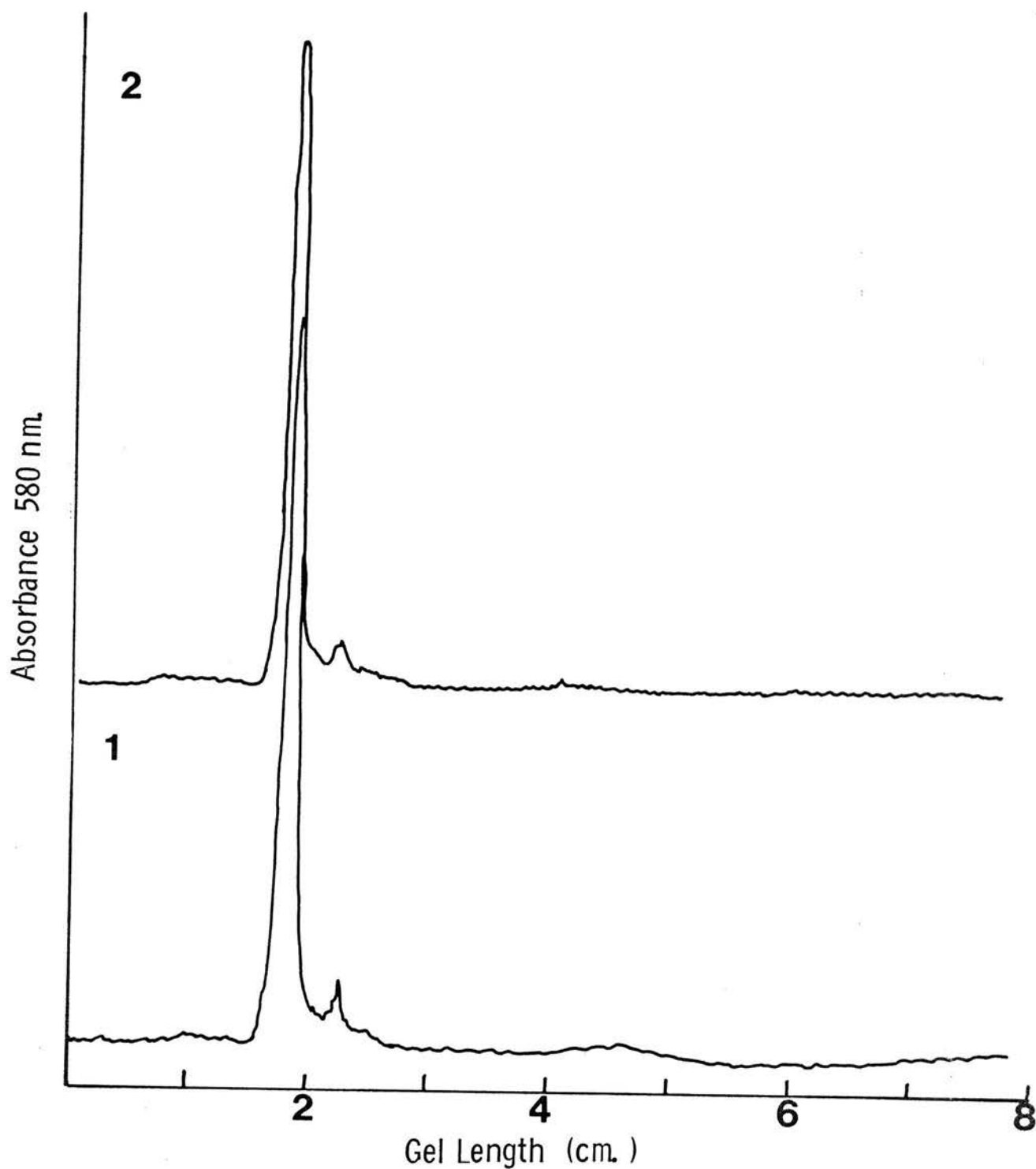


Fig. 39 Densitometric scans of acetic acid urea polyacrylamide gels after electrophoresis of:
1) rat liver apoferritin
2) the main carboxymethylated component of rat liver apoferritin eluted from Sephadex G-100 in the presence of 7 Mol/L guanidine HCl.

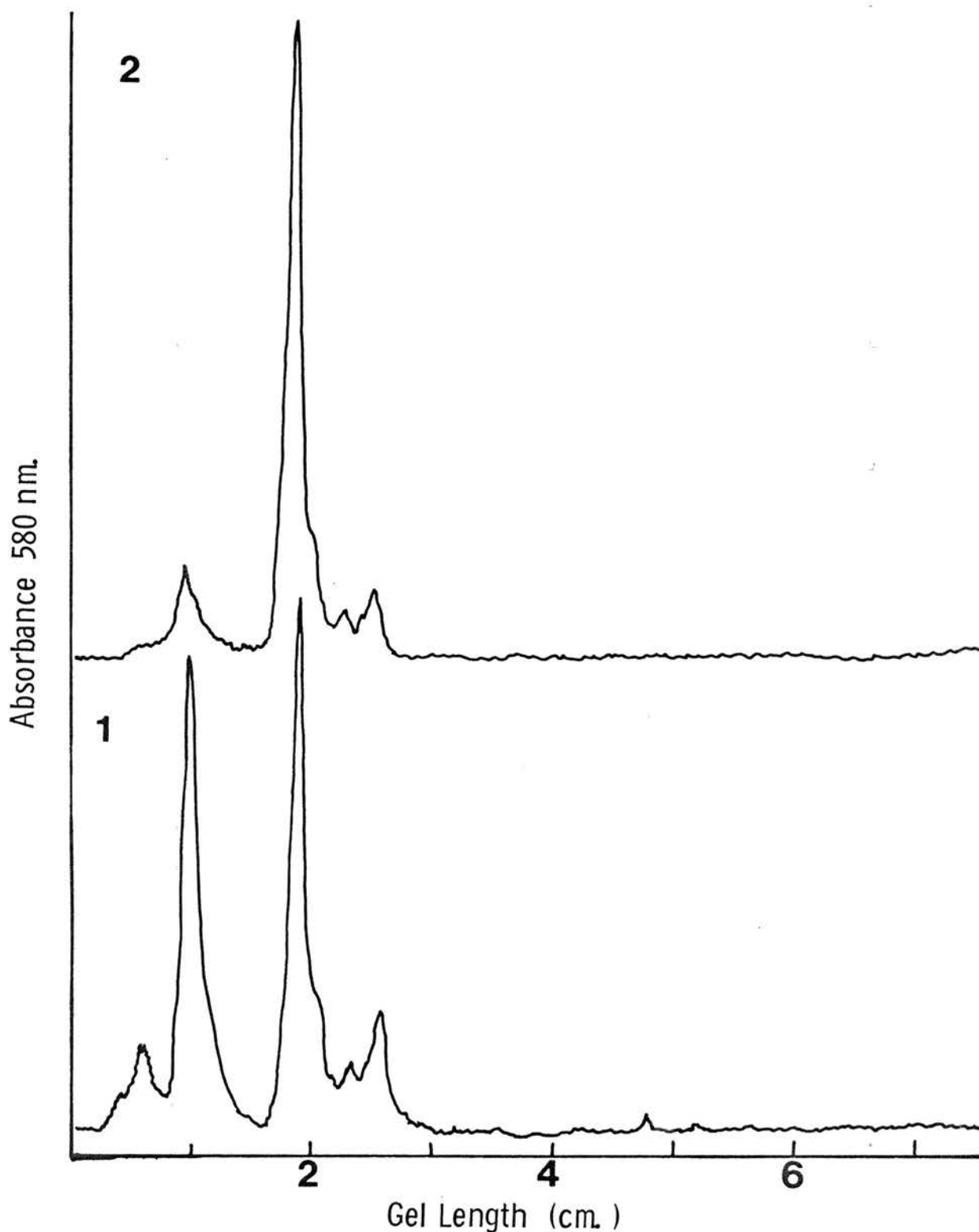


Fig. 40 Densitometric scans of stained acetic acid urea polyacrylamide gels, after electrophoresis of ;

- 1) the main uncarboxymethylated component of rat liver apoferritin eluted from Sephadex G-100 in the presence of 7 Mol/L guanidine HCl
- 2) the same sample treated with 1% (v/v) 2-mercaptoethanol, before application to the gel.

the polypeptide (Fig. 39).

Acetic acid urea electrophoresis of the uncarboxymethylated polypeptide is shown in Fig. 40. Two main bands are obvious; a slow migrating band (with a similar mobility to the faint band in Fig. 38) and another representing the dissociated polypeptide. There are also several faintly staining subsidiary bands. When the sample was treated with 1% (v/v) β mercaptoethanol (Fig. 40) the slow moving band disappeared and the amount of the faster component was greatly increased. One could conclude from this observation, that in rat liver apoferritin, intermolecular disulphide bonds may form between dissociated uncarboxymethylated polypeptide chains giving rise to a slow moving oligomeric species. This contrasts with horse and human spleen apoferritin where the uncarboxymethylated polypeptide chains appear to have a tendency to form intramolecular disulphide bonds.

(d) The 12-13,000 and "6-7,000" Molecular Weight
Polypeptides

Small quantities (~ 0.5 mg) of the two smaller polypeptides were obtained from the G-100 column at the same time as the larger polypeptide during the experiments described in this section. These smaller polypeptides

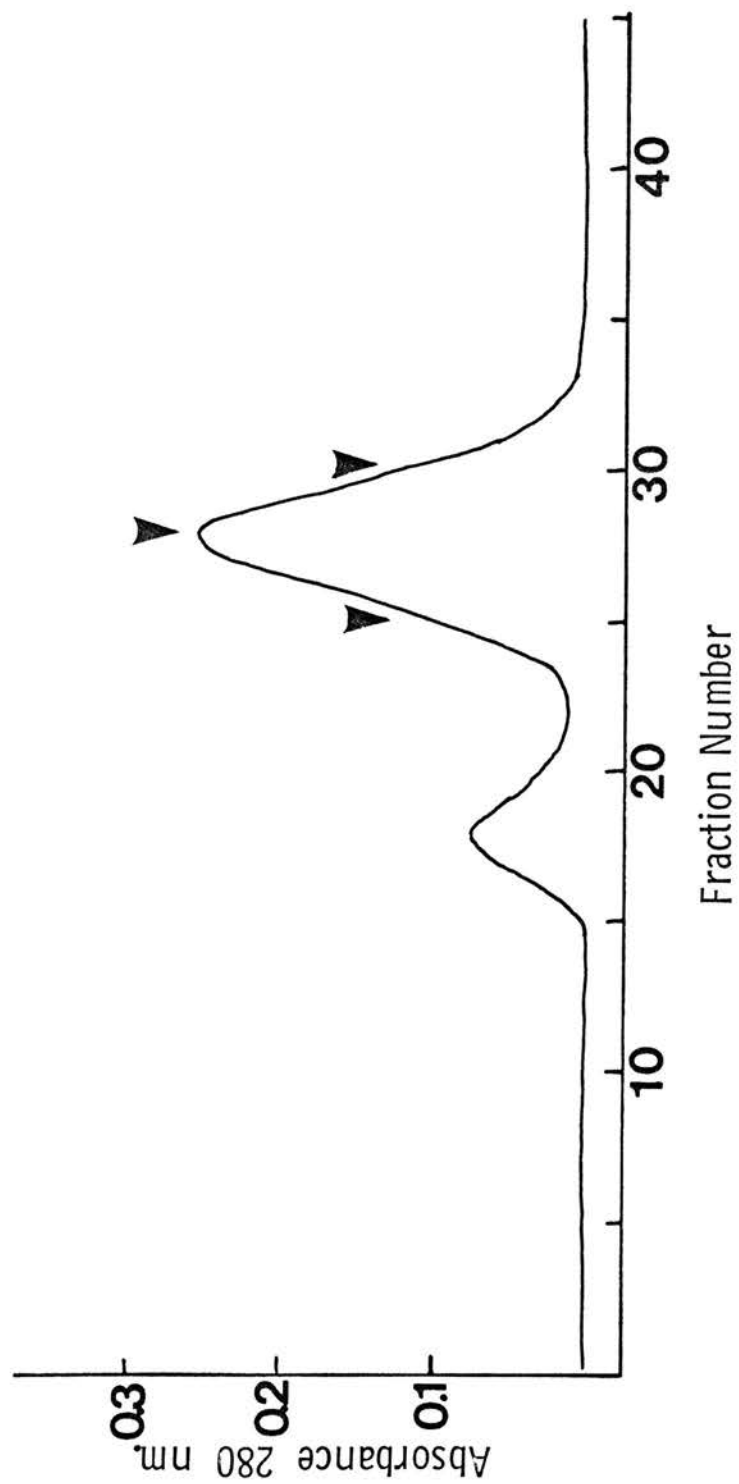


Fig. 41 The elution profile of horse spleen apoferritin, after gel filtration on Sephadex G-100 equilibrated in 0.1 Mol/L glycine HCl buffer pH 1.6. The arrows indicate the fractions which were analysed by acetic acid urea electrophoresis.

from the three tissue sources used were then subjected to acetic acid urea electrophoresis. The 12-13,000 molecular weight species appeared as a very diffuse protein staining band half way down the length of the gel. The smaller species did not stain at all on these gels. Because of the difficulties encountered in obtaining sufficient quantities of these components for similar analyses it was not possible to ascertain why these components did not migrate as sharp bands.

Analysis by Acetic Acid Urea Electrophoresis of the
Products Eluted from Gel Filtration in Acid

An ultraviolet absorbance profile of horse spleen apoferritin eluted from Sephadex G-100 equilibrated in 0.1 mol/l glycine/HCl buffer pH 1.6 (see Chapter IV) is shown in Fig. 41. Two peaks are evident, a small absorbance peak of undissociated material at the void volume and a larger absorbance peak of dissociated material. The problem here is basically that following acid dissociation, acid urea electrophoresis shows two dissociated polypeptides whereas acid dissociation followed by gel filtration shows only one dissociated species. Of course the likely possibility, considering the closeness in size of the electrophoretically separated polypeptides, is that they are not completely resolved by gel filtration and are eluted as a single peak. Accordingly one might

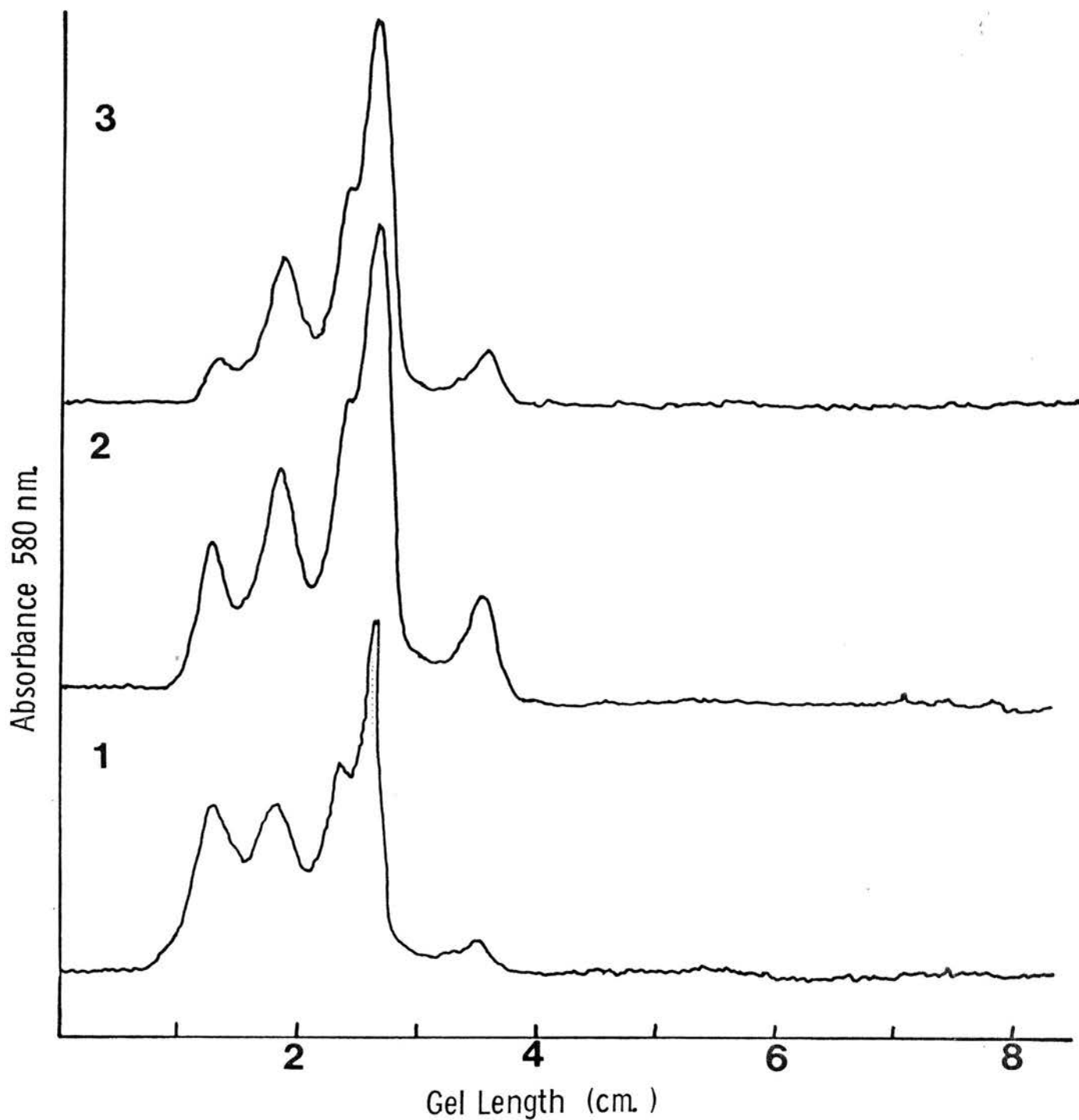


Fig. 42 Densitometric scans of stained acetic acid urea polyacrylamide gels, after electrophoresis of fractions eluted from the "acid gel filtration" on Sephadex G-100, of horse spleen apoferritin. The fractions corresponded to :
 1) the upward slope of the elution profile,
 2) the peak of the elution profile,
 3) the downward slope of the elution profile.

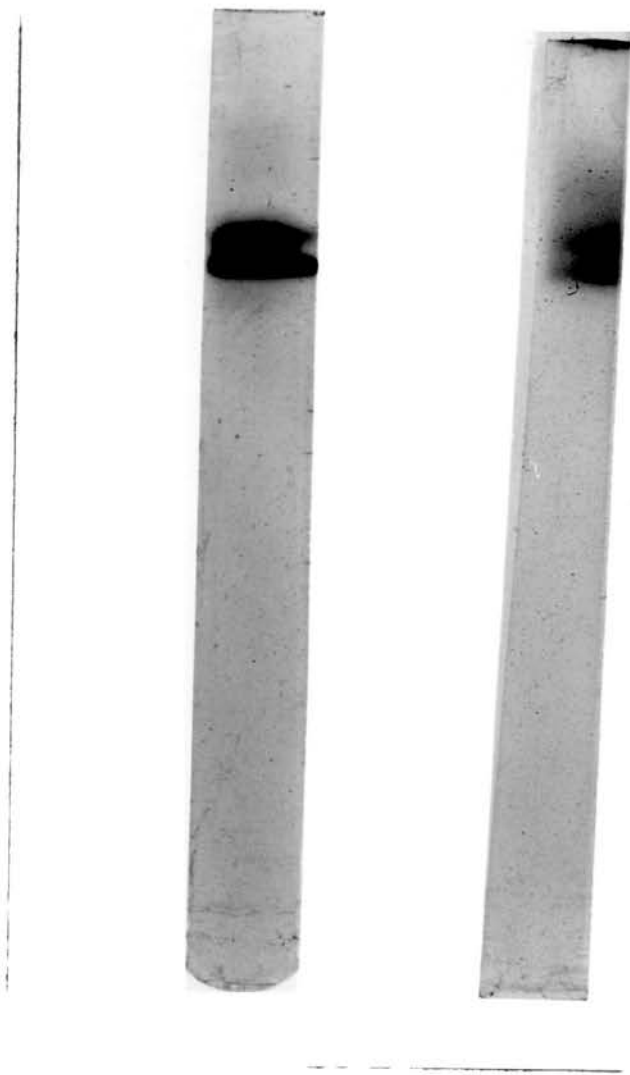


Fig. 43 Acetic acid urea polyacrylamide gels after electrophoresis of, from L to R, human spleen apoferritin and the corresponding component eluted from Sephadex G-100 equilibrated in acid buffer.

expect that fractions taken from different areas of the peak might, when analysed by acetic acid urea electrophoresis show different amounts of the two polypeptides. Fractions from the upward sloping region of the peak might be expected to have more of the higher molecular weight polypeptide than those fractions taken from the descending slope of the peak.

Gel scans of acetic acid urea electrophoresis of the three fractions taken (as indicated in Fig. 41) are shown in Fig. 42. In all three gel scans, five peaks were observed; two corresponding to the slow migrating components commonly seen on gels of horse spleen apoferritin and shown in this work to represent oligomeric species, and the three peaks characteristic of horse spleen apoferritin. The relative amounts of the three main bands did not change appreciably from fraction to fraction.

A similar series of experiments were carried out on human and rat spleen apoferritin. In each case, the acid dissociated protein was eluted from Sephadex G-100 as a single absorbance peak, and fractions from the relevant areas of the peak were analysed. Fig. 43 shows the results of the experiment on human spleen apoferritin. The two gels shown are acetic acid urea gels of human spleen apoferritin and one gel representative of the three fractions analysed. All three fractions gave

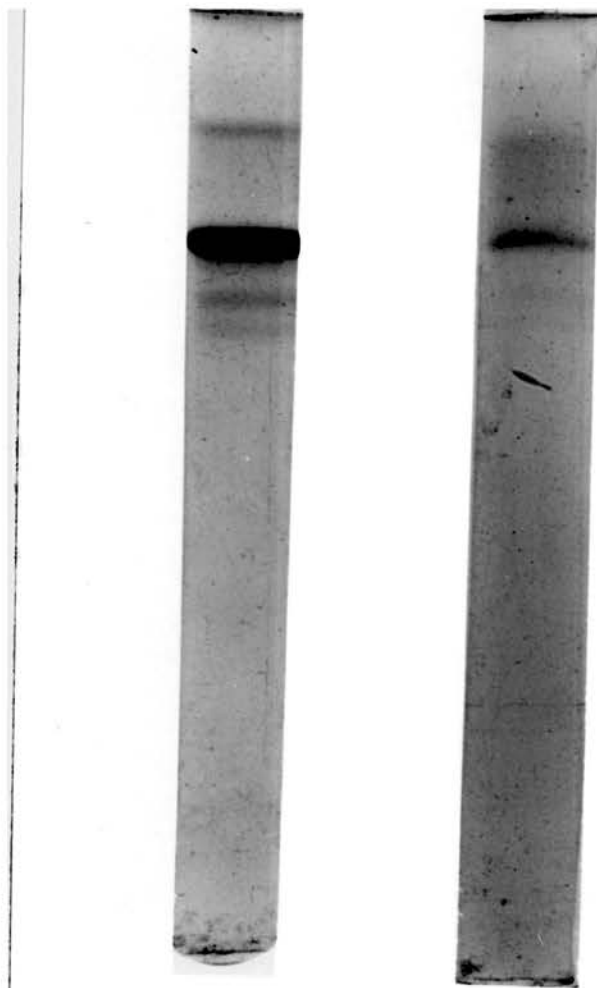


Fig. 44 Acetic acid urea polyacrylamide gels after electrophoresis of, from L to R, rat liver apoferritin and the corresponding component eluted from Sephadex G-100 equilibrated in acid buffer.

identical banding patterns, which in turn were similar to those present on the gel of the unchromatographed protein. It should be noted that in this experiment, the absence of the "15,000" band is because before application to the electrophoresis gel the samples were treated with 1% (v/v) β mercaptoethanol. The reason for this was to "tidy up" the gels by removing the oligomeric species and the "15,000" band and thus facilitate analysis of any changes in the more important dissociated polypeptide bands.

The acetic acid urea gels of rat liver apoferritin and the corresponding fractions eluted from Sephadex G-100 are shown in Fig. 44. The "whole" protein gave a single band (subsidiary bands on these gels were trace impurities in this sample) typical for rat liver apoferritin, while the eluted dissociated fractions also gave a single band. This result was somewhat as expected since the experiments on horse and human spleen apoferritin had shown that electrophoresis of the chromatographed dissociated material gave similar bands to those seen on gels of the "whole" protein.

*Analysis of the Products of Acetic Acid Urea Electrophoresis
by SDS Electrophoresis*

The results of this experiment were described in

Chapter 5 (5.3). Summarised, this experiment showed that the three horse spleen apoferritin bands observed on acetic acid urea electrophoresis represent components of different molecular weight, these being 20-21,000; 18-19,000 and an artifactual component, the "15,000" band, rather than three polypeptides of similar molecular weight and different charge properties.

6.3 Conclusion

The experiments described in this chapter which have attempted to integrate the irregularities observed when the subunit structure of apoferritin is analysed under different dissociating conditions, have shown certain trends. The products of dissociation of apoferritin in guanidine hydrochloride are identical to those dissociated by SDS. When the separated polypeptides dissociated in guanidine hydrochloride were subjected to SDS electrophoresis, components were observed with mobilities identical to those observed on SDS gels of the "whole" protein. This confirmed similar findings by Collet-Cassart and Crichton (1975) obtained at the same time as the results described in this work.

Other combinations of analytical techniques disclosed certain differences. Acetic acid urea electrophoresis of the major polypeptide dissociated in guanidine hydrochloride, and that dissociated in 67% (v/v) glacial acetic

acid, provided gels with multiple protein staining bands, although each dissociated product was eluted from Sephadex G-100 as a single component.

Furthermore, differences were observed between the human and horse spleen apoferritin and rat liver apoferritin polypeptides dissociated in guanidine hydrochloride. The two former polypeptides showed a tendency towards intramolecular disulphide bonding while the latter polypeptide tended to form intermolecular associations through disulphide bonding.

Overall, the results have shown that the dissociation of apoferritin can be classified into three types:

- (i) guanidine hydrochloride - SDS dissociation which both produced identical components. These seem to be the most powerful dissociating agents giving rise not only to a polypeptide of molecular weight 18-19,000 but also to two smaller molecular weight polypeptides.
- (ii) acetic acid dissociation resulting in the elution from Sephadex G-100 of an apparently single polypeptide type.
- (iii) acetic acid urea electrophoresis, which can resolve, without prior dissociation horse and

and human spleen apoferritin into two polypeptides
(and one artefactual component) and rat liver
apoferritin into one.

CHAPTER VII

GENERAL DISCUSSION

General Discussion

At the time when the work described here was undertaken, many opposing views (summarised in Table 1) were being published from several different laboratories, concerning the quaternary structure of ferritin. These reports differed quite fundamentally on the number and size of the polypeptide composition of ferritin. This research was undertaken on the working hypothesis that these inconsistencies could be attributed to two main areas:

- (i) the variability in the methods used in different laboratories to isolate ferritin
- (ii) the application of different procedures to analyse the purified product.

At the outset the intention was to attempt to rationalise this area of protein chemistry by isolating ferritin by three different procedures, each of which included most of the stages used in other laboratories (the exception being that of Crichton, who included a cadmium sulphate precipitation stage (Crichton, 1973), but samples from his laboratory kindly donated to us did not differ in any way from our own preparations), and by applying as many analytical procedures as possible to the question of the quaternary structure. However, rather than present results which would dramatically

change our way of looking at the quaternary structure of ferritin, by proving or disproving any of the current theories, the results instead have provided extensive confirmation of the opposing claims from the different laboratories.

The results as they relate to current theories have been discussed in the relevant chapters in the thesis. The author's intention in this chapter, is to discuss in general terms the ideas summarised at the end of chapter 6 (section 6.3).

The first interesting point to lend itself to discussion, is the source and function of the smaller components of molecular weight approximately 12,000 - 13,000 and 6,000 - 7,000 which are evident on dissociation of apoferritin in SDS and guanidine hydrochloride. Originally in an early theory, Drysdale (Adelman *et al.*, 1975) provided these entities with a functional role within the molecule, postulating that different amounts of these smaller "subunits" within ferritins from different tissue sources could be the possible explanation for isoferritins. Munro, about the same time reported metabolic and size differences between subunits (Linder *et al.*, 1974), the 13,000 molecular weight component being synthesised on free ribosomes and preferentially stimulated by iron administration. However, these latter claims have recently been retracted Zahring *et al.* (1977). Overall, within

the past two years these smaller polypeptides have received very little prominence in the literature and two of the main authorities within this area of research now seem to agree that these components have no structural significance within the molecule (Drysdale, J. W. and Crichton, R. R., personal communications).

It seems likely that these smaller polypeptides represent products of proteolysis of the large subunit. Evidence that these components could originate from the larger subunit comes from Niitsu *et al.* (1973) and Ishitani *et al.* (1975) who showed that the sum of the amino acid content and molecular weights of the smaller polypeptides equalled that of the largest, while Collett-Cassart and Crichton (1975) showed that superimposition of the peptide fingerprints of the two smaller polypeptides was identical to that of the largest.

If proteolysis occurred during the isolation procedure, two stages were likely to encourage it: the first, the stage at which the pH was lowered to 4.8 which would encourage the action of cathepsins, especially cathepsin B (Otto, 1971), and the stage at which the temperature was increased to 70°C. Experiments were carried out in association with Professor Crichton in which ferritin preparations were incubated with tissue homogenates which were assumed to contain proteolytic activity. The ferritin was repurified and analysed by SDS electrophoresis.

Although initial experiments showed an increase in the amount of smaller polypeptides after this incubation, it was not possible to show consistently, and hence conclusively that these polypeptides originated via proteolytic activity present in the tissue homogenate. Furthermore, the lack of any change in the amount of these polypeptides when the heat treatment stage was omitted from the isolation procedure (Chapter 5) casts doubt on homogenate proteolysis as their source. Finally if proteolysis did occur during the isolation procedure, then the smaller peptides formed would probably be lost during the final purification stage which involved gel chromatography.

A possibility proposed here, is that the proteolytic cleavage occurs after the final purification stage. Proteins are known to be more susceptible to proteolysis in a denatured form (Segal, 1976), and it is possible that proteolysis occurs during denaturation in SDS and guanidine hydrochloride both of which involve lengthy incubations at 37°C and at neutral pH at which temperature and pH it may be susceptible to the action of trypsin or chymotrypsin. Further evidence for this theory comes from Freedman *et al.* (1976) who have reported the presence of proteolytic activity in commercial ferritin preparations. Although commercial ferritin preparations are in general of a somewhat more impure form than those reported here (see Figures 4 & 5), it is possible that the ferritins isolated in this work may contain proteolytic contaminants

which could attack the protein once it had been rendered more vulnerable in a denatured form.

This theory could also explain the absence of these smaller components on acid gel filtration and acetic acid urea electrophoresis, both procedures which involved denaturing the protein in 67% acetic acid. In 67% acetic acid the pH of the denaturing solvent is 1.5 and although cathepsin E has a pH optimum of 2.5 (Lapresle and Webb, 1962) it is very unlikely that any proteolytic activity would occur at such a low pH.

The second main feature of the results, is the presence of a polypeptide with a molecular weight of approximately 21,000 on acetic acid urea electrophoresis which is completely absent on SDS electrophoresis. It must be repeated (cf. Chapter 5) that our data on SDS electrophoresis of apoferritin fail to confirm recent reports by Drysdale (Drysdale *et al.*, 1977) that SDS dissociation and electrophoresis can resolve ferritin into two "subunits" of molecular weight 19,000 and 21,000 although we have used similar techniques to those used by Drysdale. The inability in this work to demonstrate the two acetic acid urea "subunits" on SDS electrophoresis raises doubt to their authenticity, and speculation that they may be artefacts of that particular electrophoretic system. Until more experiments are carried out on these components, it is difficult to comment on their validity.

Assuming however that they are real components then they may represent subunits with functional differences within the molecule. One or either of these polypeptides may represent a subunit population located at the intersubunit channels (Macara *et al.*, 1973; Crichton, 1973) where oxidation and reduction of iron occurs, while the other population may have a purely structural role. This theory can be tested once the subunits involved in oxidation-reduction reactions are separated and purified, possibly by affinity chromatography.

The third main point for discussion is the difference in disulphide bonding properties between on the one hand horse and human spleen ferritins, and on the other rat liver ferritin. It has been possible to show that the unfolded SDS dissociated 18-19,000 molecular weight component of all three proteins, in the absence of a suitable reducing agent folds in on itself, and forms an entity by way of intramolecular disulphide bonding with electrophoretic properties characteristic of a 15,000 molecular weight polypeptide. This confirmed reports by Niitsu *et al.*, (1973) and Ishitani *et al.* (1975). The rat liver apoferritin "15,000 subunit" was less susceptible to the effect of reducing agent than the spleen apoferritins. Surprisingly, although this "15,000 component" is present on acetic acid urea gels of the spleen apoferritins in the absence of reducing agent it does not appear on gels of rat liver apoferritin. In fact in this system rather than

forming intramolecular disulphide bonds the rat liver apoferritin polypeptides aggregate through intermolecular disulphide bonding. This observation is difficult to interpret and is typical of several other anomalies associated with rat liver apoferritin:

- (i) on gel filtration its "subunits" elute earlier than those of the two spleen ferritins while on SDS electrophoresis its mobility is apparently identical to that of the two spleen proteins.
- (ii) on acetic acid urea electrophoresis it shows a single component of molecular weight 21,000 while the spleen apoferritins show two components of molecular weight 19,000 and 21,000.

Drysdale (Drysdale *et al.*, 1975) observed tissue differences between the acetic acid urea electrophoretic behaviour of ferritins. However his finding that the HL subunit (19,000) predominates in liver tissue does not agree with our data for rat liver ferritin, which corresponds with a polypeptide molecular weight of 21,000, to that of the H subunit reported by Drysdale to be typical of heart tissue.

In conclusion, it is felt that this work has achieved many of the aims which were set at the beginning, without establishing the final solution to what is a very complicated

problem. The many controversies associated with any discussion of the quaternary structure of ferritin have not been fully resolved. Suggestions as to the future experimental approach to the problem include the following:

the extraction and purification of the components of ferritin separated by both SDS and acetic acid urea electrophoresis, and a comparative analyses of these polypeptides involving the following techniques

- (a) amino acid analysis
- (b) peptide fingerprinting
- (c) isoelectric focusing
- (d) amino acid sequencing

Only after these experiments have been performed, will it be possible to state whether the ferritin molecule is composed of one subunit type or two. If the latter possibility is validated it is then essential to establish what functional role each subunit has within the molecule. It would not be surprising to find that one subunit type was that responsible for the uptake and release of ferritin.

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APPENDIX

Since the completion of this dissertation, two publications have appeared (Russell, S. M. and Harrison, P. M. (1978) 175, 91-104; Arosio, P., Adelman, T. G. and Drysdale, J. W. (1978) J. Biol. Chem. 253, 4451-4458) concerning the quaternary structure of ferritin. Both papers describe experiments which have attempted to clarify the controversial issues described in Chapter 2, and overlap to a large extent with much of the work described here.

In the first of these papers (Russell and Harrison, 1978) the authors reported that isoferritins vary in their ability to take up iron. An increasing tendency to sequester iron parallels a trend in isoelectric point towards acidity. The authors further discuss the conflicting views regarding the subunit structure of ferritin and quote from unpublished x-ray diffraction experimental observations comparing the octahedral crystal structure of horse liver and spleen ferritin, human spleen and rat liver ferritins. These observations suggest close similarity in the subunit size, primary structure and arrangement in the 24-subunit molecules present in the crystals, thus leading to the conclusion that it is highly unlikely that hybrid molecules containing subunits differing by as much as 2000 Daltons would satisfactorily crystallise together if the additional 2,000 mol. wt. were on the

external surface of the molecule. The authors further propose that the estimated difference of 2,000 in the subunit mol. wt. in gel migration rates could result largely from folding to more or less compact forms. This latter possibility is substantiated to some extent by the results described in this thesis, particularly those involving two dimensional acetic acid urea/SDS electrophoresis, where artifactual bands have been shown to occur due to protein chains "folding in" on themselves to form more compact structures with different apparent molecular weights.

While the first paper tended to favour the unitary hypothesis the second (Arosio *et al.*, 1978) presents further experimental evidence in favour of the "hybrid hypothesis" proposed by the same group in 1975 (Adelman, T. G., Arosio, P. and Drysdale, J. W. (1975) *Biochem. Biophys. Res. Commun.*, 63 1056-1062). They reconfirm the presence of 19,000 and 21,000 mol. wt. subunits in ferritins from rat, human and horse and report experiments involving amino acid analyses and tryptic peptide maps of these subunits showing distinct differences but also demonstrating sequence homologies. Both these subunits were identified as the primary products of apoferritin synthesis in a wheat germ lysate programmed by rat liver mRNA, thus indicating that these are products of different

genes rather than the sequence differences being due to post synthetic modifications.

It can be concluded therefore that the aforementioned controversy is still a very active one.